JAMES COOK UNIVERSITY

Exploring low molecular weight molecules produced by hookworms (*Ancylostoma caninum*) and their use as anti-inflammatory agents

PhD Thesis

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Abstract

The dog hookworm, *Ancylostoma caninum* is an experimental model for human hookworm. Relying on the ancient association of hookworms and their host and guided by the observations of the "Old friend's Theory" these parasites may be a valuable source for therapeutic discovery. This thesis is designed to study the small molecules produced by the adult hookworm with particular emphasis on identifying molecules with anti-inflammatory potential using experimental techniques not applied to any helminth previously.

To achieve this primary, aim the small molecular component of hookworm excretory/secretory fluid was extracted and separated. These fractions were separated using the small molecule techniques commonly employed in the study of ethnobotanicals. These extracts were tested in a mouse chemical colitis model known to exhibit Th1/Th17 responses, Th2 type hypertrophy, and innate immune response. This model is employed here as a broad-based bioactivity screen for immunomodulatory potential. Using these combined techniques, it was possible to identify several small molecule fractions exhibit a protective effect in TNBS mouse model of intestinal inflammation. These fractions have the potential to reveal therapeutic candidates for inflammatory disease.

During this study it was also possible to characterise the small molecule secretome of *A. caninum* using gas chromatography/ mass spectroscopy. This characterisation has been able to describe over 300 features never described for *A. caninum*. The majority of the molecules have no currently described immunomodulatory activity. This includes low-molecular weight molecules hereto undescribed. In addition to this, a number of metabolites were identified, and these could be assigned to the described pathways of the human hookworm *Necator americanus*.

It was possible to demonstrate that the small molecule milieu produced by adult dog hookworm is complex. Dog hookworm excretory/secretory material contains small molecule components that when administered to mice can prevent chemical colitis. These molecules present a novel pool of small molecules that have the potential to be used to treat inflammatory disease.

Publications relevant to this thesis

Chapter 2.

Shepherd C, Navarro S, Wangchuk P, Wilson D, Daly NL, Loukas A: Identifying the immunomodulatory components of helminths. *Parasite immunology* 2015, 37(6):293-303.

Chapter 7.

Shepherd C, Giacomin P, Navarro S, Miller K, Loukas A, Wangchuk P: A medicinal plant compound, capnoidine, prevents the onset of inflammation in a mouse model of colitis. *Journal of ethnopharmacology* 2017, 21(211):17-28.

Publications submitted

Chapter 2.

Shepherd C, Wangchuk P, Loukas A. Of dogs and hookworms: man's best friend and his parasites as a model for translational biomedical research. ACCEPTED FOR PUBLICATION; Parasites & Vectors

Publications not directly related to but relevant to my thesis

Wangchuk P, Navarro S, **Shepherd C**, Keller PA, Pyne SG, Loukas A: Diterpenoid alkaloids of *Aconitum laciniatum* and mitigation of inflammation by 14-O-acetylneoline in a murine model of ulcerative colitis. *Scientific reports* 2015, 5:12845.

Morante T, **Shepherd C**, Constantinoiu C, Loukas A, Sotillo J: Revisiting The *Ancylostoma caninum* Secretome Provides New Information On Hookworm-Host Interactions. *Proteomics* 2017.

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Data policy

Data produced from this research will be stored as per the university policy. Laboratory records will be kept with the primary supervisor. All large data files will be uploaded (when permitted) to the university data hub.

An electronic copy of this thesis wil be made available to the James Cook University library.

Findings of this research are to be published and will be available via Researchgate or ORCID (ID : 0000-0002-8108-9574)

Chapter 1
Introduction

1.1 Ancient associations meeting modern therapeutic needs:

Inflammatory bowel disease – increasing incidence and burden drives a need for new therapeutics

The autoimmune, allergic, or immune-mediated diseases resulting from chronic inflammation are typified by altered immune responses that can adversely affect any part of the body. Triggers of inflammatory diseases have revealed dysregulated immune response with an underlying genetic propensity. The incidence of inflammatory disease is on the rise globally and expected to be a significant global health burden by 2025 [1]. Specifically, Inflammatory Bowel Disease (IBD) has the highest prevalence in developed nations and is increasing as countries change their socio-economic make up and urbanise [2]. With the advent of newer therapeutics, better outcomes for patients have been achieved but the cost of treatment has increased considerably [3]. My research interests lie in the discovery of helminth-derived therapeutics which could be targeted for the treatment of IBD, with particular emphasis on small molecules that are easier to administer and likely more cost effective than many of the current protein therapeutics (biologics).

1.2 Treatments for IBD – treatments not cures

IBDs are the group of inflammatory diseases of the alimentary tract that include both ulcerative colitis (UC) and Crohn's disease (CD). Individuals that suffer from UC and CD experience a variety of debilitating symptoms including diarrhoea, cramping, pain, malabsorption, bloody stools, septic episodes, and increased risk of carcinoma. The economic burden (both direct and indirect) for these individuals and society is considerable [4]. While the clinical presentation of these two diseases can be similar the immunological profile of CD and UC is quite different, as summarised in Table 1.1. Essentially CD is a T

helper (Th) 1/Th17 dysfunction while UC is a modified-Th2 type hypertrophy. It is worthwhile highlighting that the TNBS colitis model utilised as a screening platform here clinically presents as an acute bout of UC. However, the cytokine response is a mix of Th1/Th17 with a Th2 type patchy hypertrophy (Table 1.1) reflecting neither the cytokine profile of UC or CD. This reduces the utility of TNBS as a mechanistic model but provides a broad-based screening for a large number of therapeutic targets.

	Ulcerative Colitis (UC)	**Crohn's Disease (CD)**	**TNBS Colitis TNBS/Ethanol**	**Ref**
Immunological Basis	Atypical Th2 response	Th1/Th17 mediated disease with implicated innate immune responses	Th1/Th17 with a Th2 type patchy hypertrophy	[5, 6]
Disease ameliorated by	Blocking T cell responses	Blocking IL-12p40 or TNF	Anti-IL-12, Anti-TNF and IL-10	[7, 8]
Cytokine response				
Il-1β	↑		↑	[9]
IL-2		↑	↑	[5, 10]
IL-4	±↑	↓		[10]
IL-5	±↑↑	↓		[10]
IL-6	↑	↑	±	[5, 9, 11]
IL-8	↑		↑	[12]
IL-9			↑	[13]
IL-10	↑↑			[11]
IL-12p40		↑↑	↑↑	[5, 9, 11]
IL-13	↑↑			[14]
IL-17A	↑	↑	↑ (chronic)	[5, 11, 15]
IL-18	↑		↑	[9]
IL-21	↑	±		[11]
IL-22	↑	↑		
IL-23	±↑		↑	[9, 11]
IL-27		±↑		
IL-33				
IFN-γ	↑	↑↑	↑↑	[5, 7, 11]
TNF	↑	↑	↑↑	[9, 11]
TGF-β		↑		[11]

Table 1.1 Comparison of cytokine responses in the inflammatory bowel diseases Ulcerative Colitis and Crohn's Disease, and the TNBS chemical colitis mouse model. Arrows indicate increased (upwards facing) or decreased (downwards) expression of the cytokine in the respective human conditions and mouse model.

Current treatments for IBD include non-specific anti-inflammatory drugs (aminosalicylates and corticosteroids), immunosuppressant drugs (Azathioprine, Cyclosporine, Methotrexate),

and monoclonal antibody biologics (proteins) that dampen inflammatory responses by targeting tumour necrosis factor (TNF) such as infliximab, adalimumab and golimumab, or anti-integrin biologics such as natalizumab and vedolizumab. In addition, antibiotics, nutritional supplements, and pain relief are used to manage the symptoms of IBD. The final treatment option presented to patients is surgical removal of the affected section of colon. It is worth noting that none of these options are curative. Ideal treatments would be well tolerated, easy to administer and have low complication rates.

Indications are that the initiation of IBD is multi-faceted; involving environmental factors, genes and immune status with a growing recognition that due to this complexity, individualised treatment is the most effective approach in management of IBD [16]. It is due to this complex interplay that IBD can be refractive and treatment can have serious side effects. For example, anti-TNF biologics are contraindicated in individuals with tuberculosis, fungal infections and hepatitis B as well as being implicated in the formation lymphomas and cancers because they alter the ability of the patients' immune system to respond appropriately when faced with infections, as well as the ability of the adaptive immune system to function effectively.

Better understanding of the underlying pathological processes of IBD has meant that newer therapies can be designed to target upstream initiators of inflammation. The focus on the T cell family cytokines, leukotrienes and signalling molecules associated with IBD have produced a number of therapeutic options [16]. For example, targeting the IL-12 family, specifically the p40 subunit of IL-12/23 is associated with reduction in Th1/Th17 driven pro-inflammatory responses which can be used to treat both UC and CD [17]. Currently, a phase 3 trial is recruiting for moderate to severe UC using ustekinumab which specifically targets IL-12/23p40 (https://clinicaltrials.gov/ct2/show/NCT02407236) and phase II trials for CD are being conducted [18]. There is considerable effort to generate biologics that can ameliorate inflammatory disease that in turn can be tailored to an individual's cytokine/immune dysfunction.

There are a number of issues with biologics. Infusion reactions to biologic therapeutics are considered a significant risk of therapy [19, 20]. Treatment regimens are expensive and difficult to administer; generally, patients have to be admitted to hospital for treatment.

These drugs must be administered by injection and closely monitored by highly trained individuals. Biologics are expensive to make and must be stored and transported appropriately. Comparing current cost of treatment of Crohn's disease, biologics cost 45 USD/day compared with 2 USD/day for traditional treatments [21]. This is seen for treatments of other inflammatory bowel diseases with costs in the thousands per year for biologics and hundreds of dollars for traditional treatments like steroidal drugs and methotrexate [22]. In Europe, biosimilars used for the treatment of luminal Crohn's disease such as infliximab can cost €34,500-€77,062 for five years of treatment per quality adjusted life years while a combined therapy of infliximab-adalimumab can cost up to €206,226-€363,232 for the same period [23]. The characteristics of biologics do not make them suitable for use in developing nations or remote regions. As such, the specific focus of this thesis is the discovery of targeted immunomodulatory small molecules tailored for safety and efficacy by millennia of host-parasite co-evolution that could be developed as novel, cost effective, and efficacious treatments for IBD.

1.3 Drug discovery – ancient associations outclass modern drug discovery methodologies

Small molecules are good candidates for drugs. According to Lipinski's rule of five [24] there is a sweet spot of size, solubility, acidity and permeability which determines maximum bioavailability of a molecule, or it's "druggability". Biologics fail miserably on the Lipinski rule; they are very large hydrophobic molecules that have low bioavailability [25]. Ideal treatments for IBD would be orally available, well tolerated and targeted to the specific tissues affected. With this aim, my research explores the potential of novel small molecules sourced from ancient associations. Using a mouse colitis model as a bioactivity screen it is possible to rapidly assess crude extracts, sub-fractions of mixtures and individual molecules for their immunomodulatory potential.

Modern drug discovery methods have proven to have low success rates. *In silico* computational drug design is limited due to convergent algorithms that are partly based on Lipinski, as well as our understanding of structure-activity-relationships (SAR) and the pathogenesis of the disease process. The result is a limited palate of chemical structures from which therapeutics are generated and a very low conversion rate from predicted

structure to drug candidate [26]. These molecules when generated can prove to be toxic and have low bio-acceptance [27]. To counter these problems *ad hoc* screening of small molecules sourced from biological sources (plants, animals, sponges, fungi etc.) has been attempted. Molecules from biological sources have highly diverse and novel structures. The downside is that novel molecules may be difficult to synthesise. Unsurprisingly, using an untargeted approach has resulted in very few candidates that have been successfully developed as drugs.

Pools of molecules of proven bioactivity are the ethnobotanicals. Traditional herbal medicines have been rich sources of small molecules. There are many examples of small molecules that have been developed as therapeutics. Traditional herbal medicines have been by far the richest source of small molecule therapeutics represented in the modern pharmacopeia [28]. Ethnobotanicals have an added advantage in that their bio-acceptability and toxicity has been assessed at least at a crude level. Isolating individual components and screening for specific activity can be guided by the traditional use of the herb.

There are dozens of examples of traditional herbal medicines which have yielded modern drugs, often linked with their traditional use [29, 30]. Examples include; the *Chincona spp*. - Quinine (treatment for malaria); *Ephedra sinica* - Ephedrine; *Melilotus officinalis* – Coumadin; *Papaver somniferum* – Codeine; *Taxus brevifolia* - Paclitaxel; *Salix alba* – Aspirin and *Camelia sinensis* – Theophylline. The ethnobotanicals are a considered a fruitful source of novel therapeutics compared to untargeted screening of plant material or screening of molecules synthesised from *in silico* prediction [29]. Screening is often guided by the traditional use of the herbal medicine.

In this thesis I aim to exploit the techniques used to isolate and identify bioactive molecules from plants and apply them to the small molecules produced by hookworms. I have shown that capnoidine, isolated from *Corydalis dubia,* is able to prevent many of the pathological changes associated with Trinitrobenzene sulphonic acid (TNBS)-induced chemical colitis in mice [31]. This proof of concept screening of a small molecule ethnobotanical supports the use of a bioactivity screen to demonstrate the immunomodulatory potential of natural and ancient associations.

1.4 Parasites – Humans oldest friendship

Ancient associations are not limited to ethnobotanicals; parasites are the most enduring and ancient association that humans have. The soil transmitted helminths (STH), particularly the hookworms, have such a long association with humans that they are considered “heirloom parasites” [32]. It is certain that hookworms have been associated with humans since they were hominids, with an intimate evolutionary relationship that harks back at least two million years. Observations of the co-evolutionary relationship between humans and their parasites has resulted in the theoretical basis of much of the following research and is encapsulated in the “Old friends’ theory”.

In 2002, Graham Rook coined the term “Old friends” theory to describe the requirement for exposure to organisms like hookworms, microbes, and environmental factors to drive correct immune development during childhood. Absence of exposure to organisms like hookworms is associated with immune dysfunction and the development of inflammatory responses later in life [33, 34]. The epidemiological evidence supports the “Old Friends” theory and the observation that the elimination of helminths increases the risk of inflammatory disease and immune dysfunction. In 1989 David Strachan postulated the “Hygiene Hypothesis” to explain the increase in inflammatory and allergic disease due to urbanisation [35]. This has evolved into a complex multi-pronged theory with a core position which states that a healthy and functioning immune system is reliant on early exposure to diverse factors [36, 37]. A refinement of this is the “Old Friends” theory [38] where the significance of exposure to parasites in this process should be not be underestimated.

The hookworms, like many STH, actively induce tolerance by producing molecules that interact with the host immune system at the site of their parasitism. In the case of hookworms, it is their adult stage that attaches to the gut wall for feeding and breeding that is of most interest for developing IBD therapeutics. In my review [39] of the immunomodulatory components produced by helminths, I discuss the theme of altered host immune response in the context of the molecules that helminths produce. It is apparent that the excretory/secretory (ES) component produced by helminths is a complex mix of small organic molecules, proteins, peptides, glycans and lipids. We currently know little about the immunomodulatory function of the low molecular weight component of ES (LES)

however, we know that successful helminths, particularly the hookworms, induce a "modified Th2" response. Due to the limitations of omics databases and bioinformatics workflows [39], it is necessary to screen LES for defined bioactivities to identify its immunomodulatory components. In the research that follows, I present evidence that LES from hookworms contains immunomodulatory small molecules. These molecules may contribute to host tolerance and may be candidates for therapeutic development. Additionally, characterisation of hookworm LES builds upon both secretome and metabolomics databases and will hopefully trigger similar endeavours for other helminth parasites.

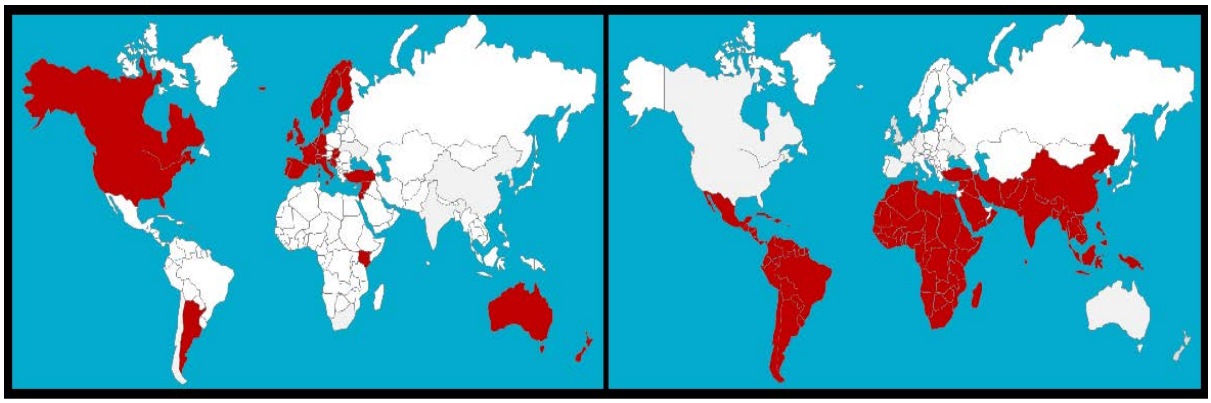

Figure 1.1. This figure shows the global distribution of autoimmune disorders (left) versus the distribution of hookworm endemic areas (right). From Molodecky *et al*. 2012 [2].

It has been noted that the incidence of inflammatory disease is highest in countries where endemic exposure to helminth is at its' lowest (Figure 1.1.) [2, 33, 40, 41]. There is epidemiological evidence to support the observation that the removal of helminths results in a dysregulation of the immune system. Large studies show that as a population urbanises and exposure to helminths is reduced, an increase in the incidence of inflammatory disease is observed [42, 43]. The implication of helminths in this process is documented in mass drug administration programs, where removal of helminths is soon followed by an increase in allergic and autoimmune disorders [44]. In a study by Kabeerdoss *et al*. of Crohn's patients in India, patients who were negative for hookworm were more likely to have Crohn's disease than uninfected patients [45]. In South Africa childhood exposure to helminths was shown to be protective against IBD [46], and in Papua New Guinea exposure to intestinal parasites protects against the development of allergic rhinitis [47]. These observations support a growing body of experimental evidence that helminths actively

produce immunomodulatory molecules that may be useful therapeutics in the treatment of allergic and autoimmune disease.

While helminth parasites are identified as a significant neglected tropical disease (NTD), with soil transmitted helminths (STH) estimated to affect over 2.2 billion people [48, 49], their effect is considered more in terms of morbidity than mortality [48]. This is because the vast majority of human helminth infections are typified by a partial activation of both the adaptive and innate arms of the immune system termed a 'modified Th2" response. That modified Th2 response consists of typical Th2 cytokines and associated cell types (IL-4, IL-5, IL-9, IL-13, mastocytosis and eosinophilia), but also features immunoregulatory components such as IL-10, TGF-β, regulatory T cells and M2 macrophages). A successful parasite is benchmarked by its ability to actively skew its host's immune system to prevent elimination and development of immunity [50].

This immune skewing results in suppression of the Th1/Th17 axis and induction of a 'modified Th2' response [51] characterised by expansion of both Th2 cells and inducible Treg expressing FOXP3, increased IL-10 levels and production of polyclonal IgE by B cells [52]. Classical Th1/17 inflammatory responses can damage worms, but at a substantial cost in collateral damage to host tissues [53, 54]. Thus, in evolutionary terms, Th2 immunity is thought to have arisen from our innate response to tissue damage, with repair responses isolating and encapsulating helminths through fibrotic processes while simultaneously resolving localised damage. Indeed, hookworms are one of the most adept infectious agents at driving regulatory responses. These strategies mean that individual parasites can colonise a host for many years while preventing the development of sterilising protective immunity and suppressing the onset of inappropriate immune responses to innocuous antigens [52, 55].

1.5 *Ancylostoma caninum*, a model for human-hookworm interaction

The dog hookworm, *A. caninum*, has been used as an animal model for the human hookworms. Phylogenetic studies highlight the close relationship between *A. caninum* and the anthropophilic hookworms *Necator americanus* and *Ancylostoma* duodenale [56-59]. In addition, the lifecycles of *A. caninum* and *A. duodenale* are very similar [60] - both have a latent stage, faecal-oral transmission, larval hypobiosis and mammary-oral infection.

Moreover, hookworms, humans, and dogs present a unique triad that makes the parasites of dogs an excellent proxy for human hookworm interaction. This is discussed at length in my review of *A. caninum* and is part of the following literature review. Due to the complicated nature of the hookworm lifecycle it is not possible to culture this parasite through all stages *in vitro*. Instead, hookworms must be harvested from the gut of the host animal for study. These parasites are then cultured in a supportive media and the ES is collected for analysis. I have been able to show herein that LES from *A. caninum* can prevent TNBS-induced colitis, but the *in vitro* culture conditions are important, and require attention if a small molecule research program is proposed.

Previous culture methods for parasites have focussed on studying parasite physiology and the effects of various anthelmintics and used a buffer-based culturing system supplemented with dog sera. Using this system it is possible to successfully sustain adult hookworms for over 80 days *in vitro* [61]. However, with the revolution in proteomics and the desire to profile ES proteinaceous components, the addition of dog sera became problematic. Removal of dog sera led to the addition of a multi-component supplement, RPMI. This supplement contains a large number of small molecules as listed in Table 2, and while suitable for the culture of hookworms and the harvesting of ES products for proteomic study; RPMI-based culturing methods are unsuitable for small molecule profiling. Individual ingredients present in RPMI are known to possess immunomodulatory activity (see Table 2). It was due to these reasons that a new culture method for the collection of ES was formulated for my research program.

Interestingly, in establishing a new culture system for hookworm omics studies, I showed that LES obtained using different minimal media displayed very different levels of anti-colitic properties *in vivo*. Changes in the small molecule milieu produced by these hookworms under different culturing conditions inferred considerable metabolic plasticity. Understanding the small molecules that hookworms produce during their gut feeding stage will enable researchers to not only understand the biology of the host-parasite interaction but also has the potential to identify immunomodulatory molecules of potential therapeutic value. From the data produced herein, I have been able to build a database of small molecules secreted by *A. caninum* that can be incorporated into other hookworm omics

databases and confirm (or refute) the existence of metabolic pathways inferred from gene/protein datasets.

1.6 TNBS chemical colitis in mice – a bioactivity screen for small molecule immunomodulatory activity

Chemical colitis is induced in mice by Trinitrobenzene sulphonic acid (TNBS) administered in an ethanoic solution by rectal enema [6]. The ethanol acts to strip the mucosa and disrupts the cells, commensal bacteria and gut contents; TNBS, a highly reactive compound, reacts with these gut components and haptenizes them. These haptens are then presented to the mouse immune system. This initiates a pro-inflammatory cytokine response involving both the innate and adaptive immune systems (Table 1.2). An acute model of TNBS – the model that is utilised here - clinically presents as an acute bout of UC, however the cytokine response is constituted of Th1/Th17 *and* Th2 pro-inflammatory responses as well as innate immune responses. While this broad immunopathologic profile is disadvantageous when trying to unpack mechanistic detail, it does present as a valuable platform to screen for immunomodulatory activity of small molecules [15]. Additional advantages of the acute TNBS colitis model are that it uses small quantities of screening material and has a rapid turnaround time of only four days.

As demonstrated throughout this thesis, the TNBS chemical colitis model provides a means of detecting immunomodulatory activity in crude LES from *A. caninum*. Using small molecule separation techniques, it is possible to determine which fractions of LES display anti-colitic protection.

RPMI Constituent	Reported Bioactivity	Reference
Niacinamide	Anti-inflammatory	[62, 63]
D-Pantothenic Acid	ND	-
Vitamin B_{12}	Anti-inflammatory	[64]
Choline Chloride	ND	-
Cyanocobalamin	Anti-inflammatory	[64]
D-Biotin	ND	-
D-Glucose	Pro-inflammatory	[65]
Folic Acid	ND	-
Glutathione (reduced)	Pro-inflammatory	[66]
Glycine	Inhibitory neurotransmitter	[67-69]
Hydroxy-L-Proline	ND	-
L-Arginine	Nitrogen donor for NO_2 synthesis, signal transduction	[70]
L-Asparagine	ND	-
L-Aspartic Acid	NMDA receptor agonist	[71]
L-Cystine	ND	-
L-Glutamic Acid	Non selective agonist, nutritive supplementation	[72, 73]
L-Glutamine	Anti-inflammatory	[74]
L-Histidine	ND	-
L-Isoleucine	ND	-
L-Leucine	ND	-
L-Lysine	Anti-inflammatory	[75]
L-Methionine	ND	-
L-Phenylalanine	ND	-
L-Proline	Induces differentiation	[76]
L-Threonine	ND	-
L-Tryptophan	Bioactivity - antidepressant	
L-Tyrosine	ND	-
L-Valine	ND	-
myo-Inositol	Improves insulin sensitivity	[77]
p-Aminobenzoic acid	Mild anti-inflammatory	[78]
Phenol Red	ND	-
Pyridoxine	ND	-
Riboflavin	Anti-inflammatory	[79, 80]
Thiamine	Anti-inflammatory	[81, 82]
L-Glutamax™ – L-alanyl-L-glutamine†	ND	-

Table 1.2. The literature-reported bioactivity of RPMI components. Bioactivity in this case is defined as pharmacological activity and not biomolecular interactions or pathways. Searches were carried out on both NCBI and Chemspider databases. ND – not reported

† Glutamax™ is included as part of RPMI complete media.

1.7 This Study

Interest in host-parasite interactions was initially borne from the extensive morbidity and mortality caused by parasites. It has become apparent that parasite success, principally gut dwelling parasites, is due to their ability to actively induce immune tolerance at the site of attachment. In this body of research, I focused on identifying a pool of small molecules of parasite origin that are derived from ancient associations of humans and hookworms. As proof-of-concept that the proposed model was valid for identifying anti-colitic small molecules, I turned to ethnobotanical compounds that were available in my lab and utilized the TNBS colitis mouse model as a bioactivity-screening tool.

Hypothesis underpinning this thesis:

The dog hookworm, *Ancylostoma caninum*, secretes low molecular weight compounds that have anti-inflammatory properties.

The first aim of this research was to assess whether the low molecular weight ES (LES) obtained from the dog hookworm, *A. caninum*, confers protection in the murine TNBS chemical colitis model. To do this a suitable culture method had to be devised for the screening of small molecules. Further to this, the nature of the protection needed to be determined by assessment of cytokine profiles. Using small molecule techniques, components of LES were extracted and purified and assessed for protective potential. I was also able to demonstrate the utility of the TNBS colitis model for determining anti-colitic efficacy of individual small molecules, as demonstrated using the plant-derived isoquinolone, capnoidine. Finally, using small molecule metabolomic techniques, the LES from *A. caninum* was characterised and annotated into known metabolic pathways.

1.8 Primary experimental aims of this research

1. Isolate LES from *A. caninum* and show protection in a TNBS colitis model.
2. Gain insights into how these molecules interact with the immune system.
3. Separate LES into fractions and determine which of these confer protection in TNBS colitis.
4. Characterise molecular components of protective fraction.

Chapter 2

Literature Review

Preamble

This chapter situates this thesis in the current research agenda and primarily draws on two review articles produced during the course of this investigation.

The first article explores the relevance of the dog hookworm as a suitable model for translational research (manuscript under peer review at *Parasites and Vectors*).

The second article explores the interest in parasite molecules as immunomodulators and as sources of therapeutic agents [39].

These articles have been edited to allow for ease of reading. Portions of these articles have been moved to relevant chapters (as indicated).

Outputs from this chapter

Of dogs and hookworms: man's best friend and his parasites as a model for translational biomedical research. Shepherd C, Wangchuk P, Loukas A. ACCEPTED FOR PUBLICATION; Parasites & Vectors

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2.1 Outline

I present evidence that the dog hookworm (*Ancylostoma caninum*) is underutilised in the study of host-parasite interactions, particularly as a proxy for the human-hookworm relationship. The inability to passage hookworms through all life stages *in vitro* means that adult stage hookworms must be harvested from the gut of their definitive hosts for *ex vivo* research. This makes study of the human-hookworm interface difficult for technical and ethical reasons. The historical association of humans, dogs, and hookworms presents a unique triad of positive evolutionary pressure to drive the *A. caninum*-canine interaction to reflect that of the human-hookworm relationship. Here I discuss *A. caninum* as a proxy for human hookworm infection and situate this hookworm model within the current research agenda, including the various omics applications and the search for next generation biologics to treat a plethora of human diseases. Historically, the dog hookworm has been well described on a physiological and biochemical level, with an increasing understanding of its role as a human zoonosis. With its similarity to human hookworm, the recent publications of hookworm genomes and other omics databases, as well as the ready availability of these parasites for *ex vivo* culture, the dog hookworm presents itself as a valuable tool for discovery and translational research.

Introduction

"His name is not Wild Dog any more, but the First Friend, because he will be our friend for always and always and always." *Rudyard Kipling*

2.2 The current hookworm research agenda

Over one-third of people on the planet harbour a parasitic helminth [49, 83]. Helminth infections are responsible for a host of morbidities that trap people in a cycle of poverty.

Moreover, parasitic diseases kill millions of people each year, primarily in developing countries of the tropics [84, 85]. Over 80 percent of helminth infections are caused by soil transmitted helminths (STH) [86]. Elimination of STH in Western society started in the early 20th century as a result of better sanitation and public sewerage programs [87]. The problem of helminth infections in developing nations was recognised, but it was not until the advent of anthelmintic drugs and the World Health Organisation initiatives of the 1950s that widespread elimination of STH was attempted [88-91].

As we approached the 21st century, rather than celebrating the worldwide demise of STH, drug failures and incomplete coverage has meant the persistence of this foe. Previously, effective drugs like mebendazole and other benzimidazole anthelmintics are now unable to completely eliminate STH infections [49, 92-99]. Historical evidence suggests that drugs alone will not result in the elimination of STH infections and the development of resistance to benzimidazoles in gastrointestinal nematodes of livestock [100-102] is a major point of concern for human STH infections, due to the reliance on albendazole for human hookworm treatment. Indications are that other means of control should be at the fore of research and development agendas [84, 103-105]. Two such avenues are the identification of new anthelmintic compounds [106, 107] and the development of vaccines that are effective against STH [84, 108]. Key to this research is a fundamental understanding of the interactions between helminths and their environments as well as the key signals that helminths require from their hosts to facilitate their extraordinary parasitic existence [91]. Characterising parasitic helminths at a deep molecular level will reveal vulnerable pathways that can be exploited by novel drugs [91, 109], and molecules of immutable vaccine antigen potential [110, 111].

Developing nations are not the only benefactors of hookworm research. Indeed, in developed countries there is an increase in the incidence of autoimmune and allergic diseases that has been linked to lack of exposure to pathogens such as STH [33]. It is apparent from epidemiological studies [43, 44, 112-114] and clinical trials [115, 116] that the immunomodulatory inputs provided by exposure to STH and other pathogens assist in the development and maintenance of a healthy immune system in humans. Characterising helminth-driven immunoregulation at a molecular level will shed light on the aetiology of

inflammatory diseases, and may uncover important disease/pathology pathways that can be targeted by helminth-derived therapeutics [39, 117].

The identification of a suitable model for the study of such a broad research agenda examining both the benefits of iatrogenic helminth infections and the pathogenesis of STH infections needs to be carefully considered. The benefit is the generation of a large amount of translatable research data coupled with an economy of scientific effort and resources. Fruitful models ideally need to be naturally occurring (i.e. not a human parasite manipulated to survive in experimental animals) and closely resemble the relationship between a STH and its human host. The human-hookworm-dog axis reflects an intimate evolutionary relationship between three organisms, a triad that presents a naturally selected model for integrated research. The dog hookworm, primarily *Ancylostoma caninum*, is discussed in this context in this review.

2.3 Of humans, hookworms and hounds – the relationship between humans, hookworms, and dogs is enduring and nuanced.

For thousands of years (estimated to be anywhere between 14,000 and ~35,000 years [118]) humans and canines have shared the same evolutionary drivers. The process of domestication has resulted in positive selective pressure on dogs to be more like humans for thousands of generations [119-121]. Genetic evidence suggests that dogs were domesticated from wolves [120, 122], with successive domestication events occurring over at least 33,000 years [118]. Importantly, dogs accompanied humans over the threshold from hunter gatherers to agricultural based societies, and this close association with humans has meant that dogs have evolved to tolerate diets rich in starch [123]. Co-habitation between humans and dogs has resulted in shared nutritional, bacterial and pathogenic environments [119, 121, 124], making dogs a natural animal model for human disease [125]. Figure 2.1 illustrates the triad relationship of hookworms, humans, and canines. Sharing environments with each other increases the risk of cross-infection with each other's parasites. We know that *Strongyloides*, *Toxocara*, tapeworms, and hookworms can be passed from dogs to humans and bidirectional transfer occurs in some cases, acting as pools for reinfection.

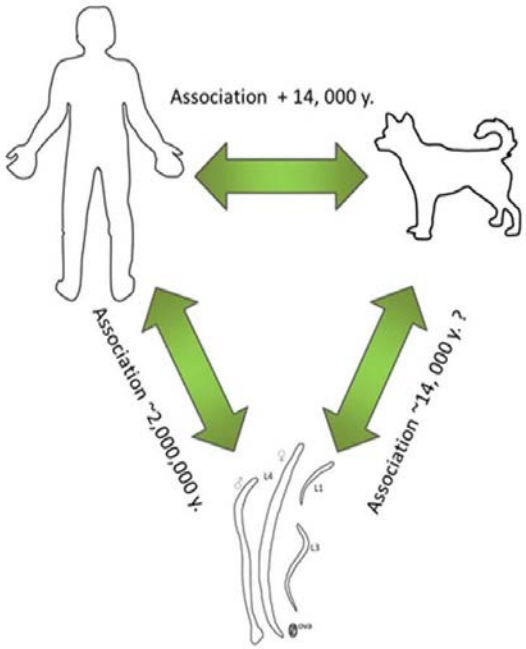


Figure 2.1. **The human-hookworm-dog relationship is defined by a long association.** Positive evolutionary pressure on canines has caused them to adapt to human environments. Hookworms have likely had conflicting immunogenic environments during cross-infection events between humans and dogs. In the ~14,000 years of association (conservative estimate) it is theoretically possible to have nearly 73,000 generations of hookworms in this timeframe.

2.4 Dogs as functional models for human disease

The parallel evolution between dog and human may have driven the predisposition to autoimmune diseases through antagonistic pleiotropy [41]. Diseases that have underlying similarities to human disease can be utilised for the study of aetiology and treatment. Genetic bottlenecks that have occurred in dog breed conformation have meant that many dog breeds are good models for both monogenic and complex genetic human diseases [48]. For example:

- Nova Scotia duck tolling retrievers develop canine systemic lupus erythematosus (SLE) which is homologous to human SLE [48]

- Osteogenesis imperfecta (brittle bone disease) in beagles and golden retrievers (polygenic disease) [49, 50]

- Several dog breeds (Pembroke Welsh corgi, Boxer, Rhodesian ridgeback, German Shepherd dog, and Chesapeake Bay retriever) develop canine degenerative myelopathy, an

amyotrophic lateral sclerosis-like disease that has similar underlying genetic basis in humans (sod1 gene) [51]

- Diseases such as narcolepsy [52], cancers [53, 54] and obsessive-compulsive disorder [55] that occur in humans have the same underlying genetic basis in dogs

Diseases like diabetes that occur in both humans and dogs are polygenic with similar underlying single nucleotide polymorphisms (SNPs) in T helper 2 type cytokines, reflecting the complex environmental and genetic sequelae in the development of this disease [56]. Similarly, Dilated Cardiomyopathy (DCM) is associated with a large number of homologous genes in both humans and canine breeds [57], making these dogs excellent models for the study of DCM in humans. These parallels between human and dog diseases mean that canine models provide identification, diagnostic and therapeutic research opportunities in a naturally occurring animal model.

The case of dogs and inflammatory bowel disease (IBD) is a little more confounding. Dogs with spontaneous IBD have similar symptoms and clinical presentation to humans with the disease [58, 59], yet the underlying basis of these diseases has not been identified. In the case of risk for gluten sensitivity in dogs there has been no link to the Major Histocompatibility Complex (MCH) genes [60], as has been found in humans with Coeliac Disease [61, 62].

Despite the established value of dog-human disease models, canine models are yet to be fully exploited as functional models for human hookworm disease. One exception is the Human Hookworm Vaccine Initiative, which used the *A. caninum*-dog model [63] for identifying molecules that could be candidates for human hookworm vaccines [3].

2.5 Hookworms are a large group of closely related organisms

In humans, the term “hookworm” is often used to refer to *Necator* and *Ancylostoma* interchangeably even though there are several distinctions as highlighted in their lifecycles, as described in Figure 2.2. These organisms are grouped together based on clinical presentation, the similarity of the organisms (in both appearance and lifecycle) and their success as parasites (Table 1.1.). Physically, hookworms can appear similar, with *Necator americanus* and *Ancylostoma duodenale* ova being difficult to distinguish visually.

Phenotypic similarities can mean that species that have large genetic separation can appear physically alike, for example *Ancylostoma braziliense* and *Ancylostoma ceylanicum* have identical mouthparts in the adult stage, making it impossible to accurately identify species without genetic tools [126, 127]. This means previous attributions of pathogenesis and distribution limits may be more complex than assumed, and in some cases flawed [127].

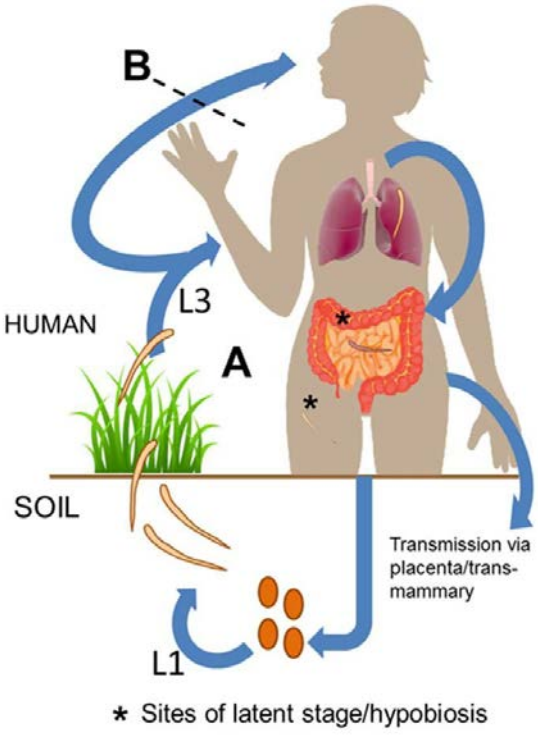


Figure 2.2 Hookworm lifecycles

Lifecycle A – Ova contained in infected faeces hatch in soil and larvae live freely for up to two moults. Third-stage larvae (L3) come in contact with skin and penetrate the epidermis. Migrating through the lymphatic and circulatory system, L3 reach the vasculature of the lung, whereupon the break through the alveolar spaces and creep up the trachea. Larvae mature *en route* to the upper gastrointestinal tract via the pharynx and become fifth-stage larvae in the duodenum. Once attached to the small intestine the sexually dioecious parasites feed, mature to reproductive capacity and mate. Eggs produced by the female worm are then shed in faeces.

Alternatively, the free-living larvae are capable of infection through the oral route.

These organisms are capable in some cases of latent stage/hypobiosis and trans-mammary or placental transmission.

Lifecycle B - does not include oral transmission. Latent stage/hypobiosis and alternative transfection routes are not reported in these organisms.

Species	Definitive Host	Human zoonosis	Paratenic host	Global incidence	Notes	Ref
Ancylostoma duodenale	Human	-	Cat, Dog	Europe, Africa, India, China, Asia, Americas	Lifecycle A* as described in [60] Hypobiosis Wakana disease in humans	[128-130]
Ancylostoma caninum	Dog	Cutaneous infection, usually asymptomatic and arrests in tissues; adult worm in gut can cause eosinophilic enteritis	-	Tropical and subtropical regions	Lifecycle A Hypobiosis	[131-133]
Ancylostoma ceylanicum	Dog, Cat, Human	-	Rodent	Asia, India, Sri Lanka, Philippines, Australia	Lifecycle A	[134-136]
Ancylostoma braziliense	Dog, cat	Cutaneous *larva migrans*	Rodent	Brazil, Africa, India, Sri Lanka, Indonesia, Philippines	Lifecycle A	[137, 138]
Ancylostoma pluridentatum	Wild cats, Jaguar, leopard	None noted	-	South and Central Americas; thought to be introduced to Florida bobcat populations mid-1950s	Lifecycle A	[139, 140]Dunbar, 1994 #3110;Forrester, 1998 #5444
Ancylostoma tubaeforme	Cat, Lynx	Cutaneous *larva migrans*	Rodent	Worldwide	Lifecycle A	[141, 142]
Ancylostoma kusimaense	Badger	None noted	Dog	Japan	Lifecycle A	[143]
Ancylostoma protelesis	Aardwolf	Unknown	-	Africa	nd	[144]
Ancylostoma somaliense	Jackal	Unknown	-	Africa	nd	[144]

Ancylostoma genettae	Genet (small African carnivore)	Unknown	-	Africa	nd	[144]
Necator americanus	Human, Gorilla	Ground itch	-	Africa, India, Asia, China, central America	Lifecycle B	[145-147]
Necator gorillae	Gorilla	Unknown	-	Africa	nd	[147]
Uncinaria stenocephala	Foxes, Wolves, Coyotes	Cutaneous *larva migrans*	Dog, Cat, Rodent	Temperate climates	Lifecycle B	[148, 149]

Table 2.1. The members of the Ancylostomatidae family and their hosts have overlapping distributions. Zoonotic disease potential, distribution and pathogenic significance are described. Lifecycles A and B are described above. nd – lifecycle is not described in the literature.

The success of hookworms as parasites hinges on their ability to be well tolerated and adaptive to their hosts. Hookworms as gut-dwelling parasites gain their nutrition from their hosts in the form of blood [62, 63]. To do this they attach to and slice through the gut wall where they feed. *Ancylostoma* is a voracious feeder, estimated to consume between 0.15-0.23 mL blood/worm/day, whereas *Necator* consumes ~0.03 mL blood/worm/day [64]. The host reacting to injury and the presence of the parasite attempts to expel the parasite through innate immune responses such as mucous production, increased peristalsis and eosinophilia [65-67]. Despite this, hookworms are usually well tolerated in a healthy host [68, 69] because they actively create an area of immune privilege around the site of their attachment by secreting a suite of immunomodulatory molecules [39, 70-72].

The hookworm genera *Ancylostoma* and *Necator* are intimately associated with the history of humanity, and it seems these heirloom parasites have evolved with humans from our primate ancestors [32, 150]. Such is the close relationship that hookworm ova have been used in archaeology to help identify migration routes of humans [32, 150, 151] and supports the intertwined immunomodulatory interactions proposed by Rook's "Old Friends Theory" [33]. In a literature survey of *Ancylostoma* and related species (Table 2.1), a number of taxonomically related hookworms were identified. These hookworms occur across a wide range of hosts with some species capable of establishing patent infection in dogs, cats, golden hamsters and humans (as is the case for *A. ceylanicum*). The common dog hookworm

(*A. caninum*) is closely related to the human hookworm species *N. americanus* and *A. duodenale.* Phylogenetic studies highlight the close relationship between *A. caninum*, *N. americanus* and *A. duodenale* [56-59, 152]. In addition, the lifecycles of *A. caninum* and *A. duodenale* are very similar [60] - both have a latent stage, faecal-oral transmission, larval hypobiosis and mammary-oral infection. The presence of a latent stage is important as these organisms have a predisposition to chemotherapeutic resistance [153] and are useful for the screening of anthelmintic drugs [154].

*A section of this review has been moved to Chapter 6 for brevity and to provide relevant background to Chapter 6 – this section has been framed by (….) *

Studies of hookworm transition through developmental stages have failed to comprehensively identify the triggers that control the penetration, migration and maturation in host tissues. Like the human hookworm *A. duodenale* [155], *A. caninum* in dog somatic tissues can enter hypobiosis, a hibernation-like state often compared to dauer formation in the free-living nematode *Caenorhabditis elegans* [156, 157]. Experimental infection of dogs with hookworms has increased our understanding of these processes. For example, experimental infection of dogs with *A. caninum* results in a greater proportion of parasites proceeding to *hypobiosis in dogs which are immunosuppressed by corticosteroid treatment*, and cessation of treatment results in resumption of parasite development, albeit in a relatively unpredictable manner [154]. The cause of this resumption is unknown, but it implies complex molecular/physiological interactions between host and parasite through all stages of development.

The propensity to hypobiosis is a valuable characteristic of a model because hypobiotic larvae are less susceptible to anthelmintic drugs [153], presumably due to their ability to slow their metabolic processes. *In vitro* studies of *A. caninum* show recovery from hypobiosis can be induced by exposure to muscarinic acetylcholine receptor agonists and cyclic GMP. This activation process results in a rapid release of infection-associated proteins [156, 158, 159], which in part mimics the host-specific signalling events seen in L3 upon exposure to host serum [159]. Very little is known about the L3 lung stage of *A. caninum* or the triggers that prompt migration and maturation of the L4 as it arrives in the gastrointestinal tract and reaches sexual maturity in the small intestine. Recent reports

show that *A. caninum* in older dogs induces a tolerogenic area around the site of attachment in the gut [160]. In addition, we know from untargeted proteomic and more targeted studies that *A. caninum* produces a number of proteins that putatively interact with immune cells and can induce anti-inflammatory responses [5, 161-164], which in turn promotes tolerance in the host.

Our understanding of *A. caninum* as a human zoonosis has also changed over time. Initially the zoonosis was defined by *larva migrans*, where the parasite was known to penetrate the epidermis but failed to successfully migrate and became trapped in the skin and underlying musculature, leading to localised irritation and pruritus [137, 165, 166]. It has since been shown that *A. caninum* is able to further its migration in humans, with occasional worms reaching the small intestine, although a patent human infection with *A. caninum* has yet to be reported. Studies in mice, a monkey and a cat have shown migration of the parasite into muscle tissues with little inflammatory response [167]. This phenomenon has also been reported in humans with migration of hookworms to muscle tissues in an individual with a large cutaneous exposure [168]. In 1994, Croese [132] described numerous cases where solitary *A. caninum* were identified in the human gut by endoscopy, discovered during clinical investigation for eosinophilic gastroenteritis [169]. These observations indicate that *A. caninum* could be a suitable proxy for human-hookworm interactions, and much can be learned about human hookworm infection by studying *A. caninum* in its natural dog host.

2.6 Omics can provide useful information but must be augmented with studies in animal models.

Omics is the collective term for the characterisation of an organism at the genome, transcriptome, proteome and metabolome levels. Increasingly, omics have been used to guide research in host-parasite biology and therapeutics discovery. This approach has been used in the case of parasitic trematodes to identify the mechanisms of drug resistance and to identify novel drug targets [170]. Metagenomics and metabolomics are the newest and most rapidly advancing 'omics' technologies, and offer under-utilised promise for understanding the molecular basis of host-helminth interactions, particularly metabolism, pharmacotherapeutic action [171] and the presence and function of commensal microbes. The limitation of comparative omics is that often there are no homologs or orthologs of

helminth molecules (particularly proteins) in other organisms to assign putative function, and many of the metabolites are of unknown origin and function. For example, there are over 700 transcripts in the *A. caninum* transcriptome encoding for proteins with secretory signal peptides with no putative function assigned [172]. Unannotated genes in parasites are of interest due to their likely role in governing a parasitic existence [173]. To augment omics databases to allow for meaningful bioinformatics analyses, animal models provide invaluable information. These models provide important bioactivity data that cannot be inferred by bioinformatics alone [39].

The currently available omics information for the family Ancylostomatidae has been assembled in Table 2 and includes for comparison the free-living model nematode *C. elegans* and the filarial nematode *Brugia malayi*. By using comparative tools it was possible using these databases to identify genes putatively associated with parasitism by comparing the different transcriptome patterns of the three helminth species [172]. Mining of assembled proteomic databases can help to identify proteins, but often the target protein is of unknown function. For example, using a shotgun proteomics approach, Mulvenna *et al.* [161] identified 105 *A. caninum* ES proteins (increased recently to 315 putative proteins in our laboratory [174]). Based on gene ontology alone, many of these proteins are of unknown function [161, 174].

Current databases for helminth omics, particularly hookworm, are incomplete. Only *N. americanus* has a published genome [175], and only *A. caninum* has a comprehensive secreted proteome from the adult worm [161], although the somatic adult worm proteome has been reported from *N. americanus* [175]. The plasticity and adaptability of hookworms further accentuates the deficit in bioinformatics data. Significant subpopulation variation of *A. caninum* has been observed [176], and it has been speculated that this variation may indicate speciation events. In addition, these subpopulations could reflect differences in infectivity for *A. caninum* [177]. The analysis of restriction fragment length polymorphisms (RFLP) of helminths from India and Vietnam supports the observed phylogenetic relationships but also identifies unclassified *Ancylostoma* species [149, 178]. This pattern is also observed with *N. americanus* [179, 180]. The source of hookworm material used to generate different omics databases is usually restricted to small populations, often passaged through unnatural hosts, and different populations are frequently used to compile different

datasets (see Table 2.2). In conjunction with the known plasticity of hookworm species, these factors may diminish valid comparisons across databases. By using *in silico* techniques it is possible to use comparative bioinformatics to combine proteomic and transcriptomic databases to determine metabolic pathways [181].

Organism	Genome	Transcriptome	Proteome/Secretome	Metabolome	Ref
C. elegans	X	X	X	X	[182-191]
N. americanus	X	X	X	–	[175, 192-198]
A. duodenale	–	–	–	–	–
A. caninum	X†	X	X	–	[161, 172, 174, 193, 199-201]
A. ceylanicum	X	X	–	–	[199, 202]
A. braziliense	–	X	–	-	[203]
Brugia malayi	X	X	X	–	[90, 172, 204-209]

Table 2.2 The current omics status for the Ancylostomatidae, the free-living model nematode *Caenorhabditis elegans*, and the filarial nematode *Brugia malayi*. Genome and transcriptome progress for Ancylostomatidae can be monitored on the Gold-genome online database (https://gold.jgi.doe.gov/distributiondata?domain=EUKARYAL&rank=family&group=Ancylostomatidae.)

Transcriptome, proteome and metabolome database updates can also be monitored at http://www.genome.jp/kegg/kegg1.html and www.wormbase.org.

†Data collection still in process.

X Omics data available

– Omics data not available

Combining omics data with functional data from animal models is a very powerful tool to identify critical physiologic and metabolic pathways. Helminth genomes are often poorly annotated, which can result in missed identifications of novel pathways in parasites. For example, it is only from metabolic labelling of intermediates of the *Leishmania* parasite promastigotes that it was possible to show that the parasite pooled reservoirs of essential molecules for the TCA cycle through the fermentation of succinate [109]. This is a novel pathway that could potentially be used as a target for anti-parasitic drugs and provides an understanding of how parasites cope with changes in their environment. The metabolome of *Entamoeba* during encystation shows the presence of polyamines [210], an unexpected finding given that the enzymes responsible for polyamine biosynthesis are not annotated in

the genome [211]. Eicosanoids in helminths are likely produced by novel pathways, as bioinformatics searches for the genes encoding homologues of the mammalian enzymes in this biosynthesis pathway have not been identified [212]. Given these examples, and the adaptability of the hookworm and its phenotypic plasticity, metabolomics profiling is likely necessary to uncover novel pathways and molecules used by these parasites [213].

2.7 Animal models of hookworm interaction

One of the major issues with the study of hookworms is the inability to culture these parasites through all developmental stages of their lifecycle *in vitro*. To this end, animal models are employed. The ideal model for human hookworm infection should reflect the pathophysiology of the helminth-host interaction and be experimentally accessible. Current models all have limitations and many animal experiments are considered "under-powered" [214] due to the ethical restrictions on animal experimentation numbers. A further restriction on models is the availability of immunological reagents for a given species. Reagents are readily available for humans, mice/rats, and to a lesser extent dogs and hamsters, but are more restricted for cats, which only have a limited palette of immune reagents. Moreover, human hookworms do not generally reach maturity in mice or rats [50, 215], precluding functional studies using genetically modified hosts.

There are three main experimental animal models commonly used as a proxy for gastrointestinal (GI) nematode infections of humans; mice/rat, hamsters and dogs as summarised in Table 3. There have been attempts to establish hookworm-primate models using marmots [216] however these models have been constricted by experimental cost [217]. The two benchmark murine models used for STH infection discussed in this review are *Heligmosomoides polygyrus bakeri* and *Nippostrongylus brasiliensis*. *Trichuris muris* is homologous to the human whipworm *Trichuris trichiura*, and while it is a STH it is not within the scope of this review. The rat is the natural host of *N. brasiliensis* and is considered a good model for human hookworm infection, particularly due to the similarities between the *N. brasiliensis* and *N. americanus* secretomes [218]. However, there are some features of the *N. brasiliensis* life cycle in rodents that are dissimilar to human hookworm infections, particularly the rapid transit time from percutaneous infection to reaching the gut, as noted

below. *N. brasiliensis* is not a natural parasite of mice, although it can complete its life cycle in them.

Animal model	**Naturally occurring Host-Parasite**	**Similar Lifecycle cf. *Necator/ Ancylostoma spp. in humans***	**Infection model**	**Model used to study**	**Regulatory Restrictions**	**Ref**
(*Mus musculus*)						
Heligmosomoides polygyrus bakeri	YES	NO (no lung stage)	Chronic helminthiasis	Pathophysiology of infection, immunological studies, vaccinomics	-	[219-225]
Nippostrongylus brasiliensis	NO	YES	Rapid expulsion	Immunology of helminth infection, Pathophysiology of infection	-	[218, 224, 226-229]
GOLDEN SYRIAN HAMSTER (*Mesocricetus auratus*)						
Ancylostoma ceylanicum	NO	NO (infection by oral gavage)	Rapid expulsion	Vaccinomics, Anti-helminthics	Hamsters not permitted in some countries (e.g. Australia)	[230-232]
Necator americanus	NO	YES	Chronic helminthiasis	Pathophysiology of infection, immunological studies, vaccinomics	Hamsters not permitted in some countries. Ova transport restricted in US	[233-235]
BEAGLE[†] (*Canis lupus familiaris*)						
Ancylostoma caninum	YES	YES, including hypobiosis	Acute and chronic helminthiasis	Pathophysiology of infection, immunological studies, vaccinomics	Ethical considerations	[236]

Table 2.3. **The major animal models used to study human hookworm infection.**

[†]Alternative sources of *A. caninum* can be from naturally infected dogs in endemic areas.

While *N. brasiliensis* is less closely related phylogenetically to human hookworms than are other *Ancylostoma spp.*, it is often used to study the pathophysiology and immunology of STH infection. One of the major differences between human hookworm infection and *N. brasiliensis* infection in mice is the self-cure phenomenon that occurs. Within two weeks of infection, mice expel *N. brasiliensis* and develop protective immunity that is dependent on $CD4^+$ Th2 cells. Indeed, this model has been used to study the development of host protective immunity and the *in vivo* regulation of Th2 responses in general [237-239]. One of the advantages of this model is the minimal inter-animal variation, however, its lifecycle is abbreviated and is not characterised by the chronicity of human hookworm infection.

As a natural parasite of mice, *H. polygyrus* is often chosen to study the pathophysiology and the immunology of intestinal nematode infections [220, 223, 237]. While taxonomically related to human hookworms, *H. polygyrus* differs from the hookworms in that it has a much simpler lifecycle. *H. polygyrus* does not have a lung stage and exhibits an enteric lifecycle with a faecal-oral infection route. However, like human hookworm infection, *H. polygyrus* establishes a chronic infection in its definitive host. As such, this model has been used to extensively characterise the Th2-skewing and immunoregulatory properties of GI nematodes [240], and ultimately resulted in the introduction of the term “modified Th2 response” [241].

The mouse models of *N. brasiliensis* and *H. polygyrus* have been used to decipher the innate and adaptive immune responses responsible for worm expulsion and the development of chronicity [242]. Both parasites drive a modified Th2 response typified by IL-4, IL-5, IL-9, IL-13 and IL-10 which mediates eosinophilia, mucosal changes such as mast cell hyperplasia, mucus production and IgG1 and IgE production [53].

One of the most important breakthroughs in the development of animal models for human hookworm infection was the establishment of patent infections in golden Syrian hamsters (*Mesocricetus auratus*) with *N. americanus* and *A. ceylanicum*. Both models however are “labours of love” and require considerable time to establish because they generally rely on immunosuppression with steroids, at least in the early phases of model establishment [233, 243, 244]. Immunosuppression with steroids of the golden Syrian hamster allows *A. ceylanicum* and *N. americanus* infections to reach patency [231, 233], thereby providing

material for laboratory analyses including genomic, transcriptomic and metabolomics studies. The *A. ceylanicum*-hamster model [245] has been utilized for testing vaccine efficacy, understanding mechanisms of adaptive immunity, and testing of anthelminthic drugs, often as a model for human infection with *N. americanus* [215, 246]. These interpretations have been supported by observations in small scale studies in humans [247-249].

The *N. americanus*-hamster model was first established by Sen and Seth in 1970 [250], and tailored as a model for testing drugs and vaccines by Jian and others in 2003 [233]. Importantly, the *in vivo* generation of adult-stage *N. americanus* enabled the sequencing of the adult worm transcriptome [192], and ultimately contributed material to the sequencing of the genome in 2014 [175]. It should be noted that *N. americanus* was adapted to hamsters through 100 generations of selective pressure. The ability of a complex multicellular pathogen to adapt to a new host within 100 generations reflects a high evolutionary rate, also observed in *A. caninum* and reflected by high DNA polymorphism [172]. We currently do not understand the molecular basis of the adaptation of *N. americanus* to the golden hamster. A comparative analysis of mitochondrial cytochrome oxidase genes from *N. americanus* obtained from hamsters and natural human infections suggests a severe genetic bottleneck in hamster-adapted *N. americanus*, which may limit the utility of this model [251]. To date there has been no investigation of the differences in genomes between *N. americanus* sourced from hamsters and those from the native human host. The 'omics' information garnered from such a comparison would be useful to highlight those genes related to host adaption and could also enhance our understanding of the metabolites produced by a system in response to changes in the host and an environmental stimulus.

There are a number of caveats to consider in the hamster model of human hookworm infection. Interpretation of data generated in the *N. americanus*-adapted hamster model may be misleading when applied to human hookworm host-parasite biology due to the undefined changes discussed above. In addition, the model using immunosuppressed hamsters and *N. americanus* is still employed [198, 252], so care should be taken when interpreting results and drawing parallels between the two models. Another problem with the hamster model of *N. americanus* infection is its restricted geographic use; for example,

hamsters are a prohibited laboratory species in Australia due to strict quarantine restrictions [55]. This limits its ability to be used in comparative and translatable studies universally. Logistically, the hamster is a small animal so the parasite load that the animal can tolerate is low when compared to a human or larger animal such as a dog. Moreover, adult *N. americanus* do not reach full size in hamsters [233], even though they do become fecund and infections are patent. A further weakness of this model is that the small numbers of adult parasites that can be supported in hamsters are rapidly expelled compared to the chronicity that develops in human necatoriasis [244].

2.8 The canine-hookworm model

The gut-dwelling, blood-feeding stage of *A. caninum* is well described at biochemical, transcriptomic and immunological levels as discussed here. The Excretory/Secretory (ES) component of *A. caninum* (AcES) at the host-parasite interface has been described, with over 200 proteins identified in 2009 [161, 174], many of which were ascribed to either feeding [253-255] or putatively evading the host and inducing tolerance [160, 162]. The immunomodulatory nature of AcES has been demonstrated [5, 256] with an increasing interest in translational applications for selected recombinant versions of AcES proteins [164, 253, 257-259]. In addition, possible mechanisms of drug resistance [96] and hypobiosis [167] have been studied in this model. The availability of such an accessible and defined model is attractive for a gamut of research agendas.

A major advantage of the *A. caninum*-dog relationship as a model for human hookworm infection is that it is naturally occurring, and moreover, *A. caninum* and *A. duodenale* are very similar morphologically and genetically. Due to the larger size of the animal, dogs are more suitable for recovering larger quantities of adult hookworms for laboratory studies than are rodents. From an individual dog it may be possible to recover thousands of adult worms, allowing for comprehensive studies to occur from a single collection. One of the major objections to the use of dogs as animal models however is the ethical concern around their experimental use; this, combined with the expense of keeping such a large animal for research purposes, often limits the use of the *A. caninum*-canine model. Attempts have been made to create an *A. duodenale*-dog model for research purposes. Using immunosuppression with prednisolone it was possible to achieve patency within 6

generations of beagle [260]. In later experiments *A. duodenale* was maintained in laboratory-raised beagles for 30 generations using decreasing amounts of prednisolone, and adaption to the dog was presumed due to observed decreases in the pre-patent period for the parasite, however a fully permissive model was not achieved due to the prohibitive resource demands of the project [236]. This is a common theme with large animal models.

Puppies usually harbour the heaviest burdens of *A. caninum*, and worm numbers (or at least eggs in the faeces) tend to diminish over time. An efficacious hookworm vaccine based on irradiated parasites was developed for dogs but was discontinued soon after reaching the market due to its poor shelf life and low uptake by pet owners [261, 262]. The dog hookworm vaccine was based on radiation-attenuated L3 [262], and although the protective antigenic components were not identified, two immunodominant L3 secreted antigens were described from dogs that received the vaccine [263]. Understanding the mechanisms by which vaccine-induced immunity to the dog hookworm develops could inform efforts to develop a subunit human hookworm vaccine. Such studies have guided antigen discovery for human hookworm vaccines [264], and even underpin proposed human trials with irradiated *N. americanus* L3 (AL, unpublished).

It is our experience that the dog-hookworm model can be very fruitful when *A. caninum* is sourced from areas where dogs are naturally infected, have not received recent anthelmintic therapy, and dogs are excess to requirements. These unwanted animals can result from wild dog eradication programmes, the culling of rural/remote camp or village dogs, or the sourcing of stray or unwanted dogs from residential communities in tropical and sub-tropical environments. The benefit of this approach is three-fold: 1) ethically, these animals are not experimentally infected or euthanized for research purposes, so ethical concerns on their use are minimised; 2) international partnerships between developed and developing countries to source these hookworms means that educational/research facilities can be meaningfully engaged in STH research; 3) harvesting of animals from diverse geographical areas gives power to observations as it reflects natural host-parasite interactions and natural spatio-temporal variation.

The opportunity for international partnering is emphasised in the modern research agenda as it aids capacity building in developing countries [265] that are most affected by STH. It

enables the development of collaborative long-term partnerships and stewardship over research agendas. This is particularly important in the case of human hookworm research as there are direct benefits to both developed and developing nations in terms of vaccine and drug development [266], as well as development of novel helminth-derived anti-inflammatory therapeutics [164]. Additionally, collaborating provides the ability to extend research capabilities in a time of increasing economic rationalisation and competitive grant processes. The inclusion of a broad base of expertise from immunology, parasitology, veterinary and agricultural sciences, public health and medicine is possible in these partnerships and is often aligned with current integrated approaches to research programs.

*A section of the submitted review has been removed here for brevity – the section is titled ***A. ceylanicum* as a model for human hookworms** in the submitted article. *

2.9 Conclusions

In the context of human hookworm research, the human-hookworm-dog axis provides one of the unique experimental opportunities for the identification of bioactive molecules for development of novel immunotherapeutics, subunit vaccines, and selection of drug targets. Domestication has meant that the unique evolutionary relationships between dogs and humans can be exploited as natural models for human diseases, notably from our perspective, the parasitic helminth infections of humans. Positive selection pressure and breed conformation has led to genetic restriction in canines and has meant that many diseases have the same underlying genetic basis in dogs and humans. It is likely that humans and dogs have shared parasites for millennia, making them an ideal model for the study of host-parasite biology.

Laboratory evidence shows that with the administration of immunosuppressive therapy it is possible to achieve patency of hookworm infections in a variety of unnatural definitive hosts. There are a number of available models for human hookworm infection; however, all of these models have limitations. Mouse models are universally available yet suffer from a range of dissimilarities to the natural human hookworm infections. Hamster models have an advantage over mouse models in that they are permissive for the human hookworms *N. americanus* and *A. ceylanicum*, but resistance to infection develops rapidly, unlike human hookworm infections.

A. caninum in the dog is a robust, naturally occurring relationship, and parasites isolated from animals in endemic areas have the potential to provide powerful molecular information that is unavailable from other sources and reflects naturally occurring interactions. The use of experimentally infected large animal models for such studies is fraught with ethical considerations and high economic costs. The study of natural interactions has the potential to provide more powerful observations than those of current laboratory models. Sourcing naturally infected animals provides an opportunity for international partnering and an opportunity for a diverse portfolio of translatable research outcomes. It negates many of the ethical and cost concerns rightly expressed over the housing of large mammals. By using a strong model for sourcing parasite material, it is possible to make reliable and translatable observations about hookworm host-parasite biology as well as facilitating programs focused on development of new control strategies aimed at eliminating, or at least mitigating, hookworm infection.

The next section focusses on the molecules that helminths produce that influence the host immune system and their possible roles as therapeutics.

The writing that follows is from: **Identifying the immunomodulatory components of helminths.** Shepherd C, Navarro S, Wangchuk P, Wilson D, Daly NL, Loukas A: Parasite immunology 2015, 37(6):293-303. PUBLISHED WORK, Permission to publish here has been obtained.

2.10 Preamble

Immunomodulatory components of helminths (ICH) offer great promise as an entirely new class of biologics for the treatment of inflammatory diseases. Here we discuss the emerging themes in helminth-driven immunomodulation in the context of therapeutic drug discovery. We broadly define the approaches that are currently applied by researchers to identify these helminth molecules, highlighting key areas of potential exploitation that have been

mostly neglected thus far, notably small molecules. Finally, we propose that the investigation of immunomodulatory compounds will enable the translation of current and future research efforts into potential treatments for autoimmune and allergic diseases, while at the same time yielding new insights into the molecular interface of host-parasite biology.

2.11 Introduction

The immunomodulatory properties of helminths are to a large extent attributed to the excretory/secretory (ES) molecules that they release when migrating and feeding inside their hosts [52, 117, 267]. We herein refer to the immunomodulatory components of ES products as ICH, or Immunomodulatory Components of Helminths. The secretomes of parasitic helminths are largely the subject of three main avenues of investigation - molecular biology of the host-parasite relationship, anti-helminth drug and vaccine discovery, and the discovery of novel therapeutics for treating diseases (primarily, but not restricted to inflammation) of humans, animals and plants. Given this broad scope we will not endeavour to discuss helminth vaccinomics, a topic that has been reviewed elsewhere [268, 269]. Considering the enormous disease burden of human and animal helminth infections, the identification of ICH could serve to identify targets for novel vaccines that aim to interrupt immunoregulatory processes of helminths. It has however become evident that targeting ICH for vaccines can result in atopic reactions [163, 270, 271] and more selective and strategic approaches to vaccinomics are currently being adopted [272]. In this article we will instead focus on the anti-inflammatory properties of helminths and explore the notion that therapeutic application of ICH for treating inflammatory diseases is a valid and timely approach to drug discovery guided by robust and cutting-edge science and clinical medicine. We believe that by identifying the major bioactive ICH we will not only identify novel drug leads for the treatment of autoimmune and allergic diseases but will augment research into the immunobiology and co-evolution of the host-parasite relationship.

There are five evident themes concerning ICH that emerge from the literature:

1. ICH is a complex mixture of proteins, peptides, glycans, lipids and small organic molecules.

2. Successful helminths induce a ‘modified Th2’ response in the host via secretion of ICH.
3. Many individual ICH components are pleiotropic.
4. ICH bioactivity cannot, for the most part, be predicted by current bioinformatics-based modelling.
5. The low molecular weight component of ES has received little attention to date, in terms of both characterisation and function. Nonetheless, there are a few descriptions of small molecules derived from helminths that display immunomodulatory properties and possess desirable pharmacological properties.

The targeting of potential therapeutic agents from ICH will define motifs that orchestrate fundamental processes in host-parasite biology, enable the identification of suitable host targets with high druggability, and further our understanding of how these molecules modulate the immune system in a safe manner. Indeed, the discovery of targeted, novel and effective ICH-derived treatments for the autoimmune and allergic diseases that plague industrialized nations may ultimately reveal how these inflammatory diseases develop. The field of host-parasite immunoregulation is benefiting from an accelerated momentum and unprecedented opportunities to rapidly progress findings out of the academic sphere and towards clinical development.

2.12 Background

The observed explosion in autoimmune and allergic inflammatory diseases in industrialised societies has accompanied the rapid improvement in sanitation and infectious disease control [41]. Evidence from epidemiological studies shows the importance of helminths in this process. Removal of helminths from populations in economic (and sanitary) transition results in an increase in the incidence of inflammatory diseases such as asthma, allergic rhinitis, ulcerative colitis, Crohn’s disease, rheumatoid arthritis, food allergy, type-1 diabetes and multiple sclerosis [44, 273-275]. Additionally, studies on the migration of populations from areas of poor to high sanitation have revealed an increased risk of diseases such as inflammatory bowel disease (IBD) [43]. Indeed, the children of migrants can acquire the inflammatory disease rates of the population they are born into [276-278].

Two of the theories used to explain these observations are the Hygiene Hypothesis and the Old Friends theory. The Hygiene Hypothesis proposes that exposure to pathogens during childhood (via sibling contact) is necessary for the immune system to develop and self-regulate [33, 35, 37, 279]. This lack of exposure to pathogens along with an underlying genetic pre-disposition can result in the development of autoimmune or inflammatory diseases [280]. The significance of exposure to helminth parasites in this process cannot be underestimated [33, 41, 113]. The blood-feeding helminths are long-standing parasites of humans [151] that are thought to have co-evolved with mammals [280, 281]. The Old Friends theory [38] is an extension of the Hygiene Hypothesis and supposes that the long association between host and parasite has resulted in a co-evolutionary dependence [282], where parasitic helminths rely on their hosts for nutrients and niche, and the host's immune system is primed to expect signals from helminths, including ICH [279]. Without exposure to helminths, inflammatory diseases are more likely to develop, however high parasitic burdens can result in loss of fitness [50, 279, 283] (Figure 2.3). For the development of a healthy immune system, there appears to be a requirement for exposure to helminths, while minimising the burden of excessive parasitic load.

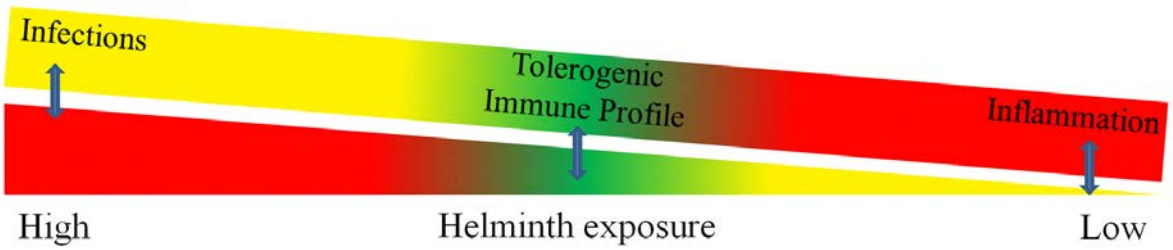


Figure 2.3. **The relationship between helminth exposure and immune response.** In areas with high helminth infection rates there is a link to increased infection risk; conversely low helminth exposure can result in a pro-inflammatory profile. The proposal that a moderated exposure to parasites results in a balanced immune phenotype underpins the key observations of the Hygiene Hypothesis and Old Friends theory and is governed by the molecules produced by parasites. Adapted from Wiria et al. [283]

Mining of ICH for therapeutic compounds has clear advantage over some of the more traditional drug development strategies, like the use of combinatorial chemistry [26] and *in silico* screening [284, 285], primarily due to a 24% success rate of taking a drug from clinical trials to market compared with 11% for new chemical entities [286]. Despite promising outcomes from clinical trials using experimental human helminth infections to treat IBD [287, 288], multiple sclerosis [115] and coeliac disease [289], there has been little interest

from big pharma in translating live helminth therapy into an industrial scale therapeutic modality. Smaller biotech companies have, however, invested in such projects, exemplified by ongoing and recently completed phase II clinical trials with *Trichuris suis* ova (TSO) for the treatment of numerous indications including IBD, multiple sclerosis and autism (http://www.coronadobiosciences.com/research-development/TSO.cfm). Despite significant progress in the clinical development of TSO and its good tolerability in human subjects [290], recent clinical trials assessing TSO for the treatment of Crohn's disease (TRUST Phase I and II) failed to meet their clinical endpoints (http://ir.coronadobiosciences.com/Cache/1500053915.PDF?Y=&O=PDF&D=&FID=1500053915&T=&IID=4308955). The notion of treating people with live helminths still poses significant hurdles in terms of public acceptability, regulatory approval and standardisation procedures. Moreover, helminths are a source of significant morbidity and mortality worldwide [55, 60]. Although low-dose experimental infections with human helminths in well-nourished individuals appears to be safe and well tolerated [291-294], the use of pathogenic human helminths is restricted in some countries, including the U.S. [235] where human studies have been limited to zoonotic helminths [287, 295]. The use of zoonotic parasites is not without its own risks, as migration paths of these organisms can be unpredictable and can result in iatrogenic disease [117, 132, 296, 297]. Additionally, unlike maggots and leeches, which are considered "medical devices", the US Food and Drug Administration (FDA) considers helminths to be a "drug", meaning that studies involving experimental infections are required to undergo the same rigorous validation as a chemical agent, including a thorough pharmacokinetic study. Table 2.4 provides a summary of live helminth treatment of inflammatory bowel disease with a notation on the status of each clinical trial.

Parasite	Natural Host	Disease Treated	Treatment Regime (time period)	Outcome	Clinical Trial Phase	Ref
Necator americanus		CD	25-100 L3 (45 weeks)	88.9 % of patients showed improvement in Clinical score index	POC	[288]
Necator americanus		Celiac Disease	5-10 L3 (21 weeks)	No significant improvement in disease	I	[291]
			20 L3 (52 weeks)	Induction of tolerance when combined with gluten escalation	I	[289]
Trichuris suis		UC/CD	2500 ova x 1 (12 weeks)	Temporary remission in 86% of patients	I	[295]
Trichuris suis		CD	2500 ova x 8 (24 weeks)	Reduction in symptoms (79.3%) Remission in 72.4% of patients	I	[287]
			7500 ova x 6 (10 weeks)	No significant improvement in disease	II	[298]
Trichuris trichiura		UC	500 + 1000 ova (3 years)	Self-reported amelioration of disease – single subject	POC	[292]

Table 2.4. A summary of experimental human helminth infections for the treatment of inflammatory bowel diseases.

CD = Crohn's Disease; UC = Ulcerative Colitis; POC = Proof of Concept; I = phase I clinical trial; II = phase II clinical trial; L3 = third stage infective larvae.

Despite the drawbacks of live helminth therapy, molecular products derived from helminths have been convincingly shown to possess potent immunomodulatory properties in a range of animal models of inflammation [299]. Identification and subsequent production of recombinant/synthetic versions of specific ICHs will likely overcome many of the problems encountered using live helminths or crude ES products as therapeutic modalities for inflammatory diseases [9, 44, 117, 300-304].

While there is a substantial body of literature showing the immunoregulatory properties of helminth ES products, there is however a great deal of variability in the preparation and

screening of ES products for activity [305]. This makes it difficult to meaningfully compare the immunoregulatory properties of ES products from different helminths. There is little consistency in methodologies for the production of ES products and methods employed when screening for ICH (Table 2.5). Moreover, many researchers exclude the low molecular weight components of ES products (peptides and small molecules) during a concentration/diafiltration step; we discuss the potential of small molecule ICH later in the review.

Species	ES Product Size	Model	Immunomodulatory Outcome	Refs
Schistosoma mansoni	0.2 µm filtered	Mouse chemical colitis model	Induction of modified Th2 response	[5, 306]
Heligmosomoides polygyrus	>10 kDa	*In vitro* co-stimulation of murine BMDC	Induction of modified Th2 response	[307]
Trichuris suis	>10 kDa	*In vitro* culture with IPEC-1 cells	↑ IL-6 ↑ IL-10 Th2 type response	[308]
Nippostrongylus brasiliensis	>5 kDa	*In vitro* co-stimulation of murine BMDC Murine asthma model	Induction of modified Th2 response	[238, 309, 310]
	0.2 µm filtered	*In vitro* suppression of murine D10.G4	Suppression of Th2 cells ↑ IL-4	[311]
Fasciola hepatica	>10 kDa	*In vitro* co-stimulation of murine BMDC	Induction of modified Th2 response	[312]
Opisthorchis viverrini	0.2 µm filtered	*In vitro* culture with H69 cells	↑ IL-6 ↑ IL-8 Pro-inflammatory	[313]
Ancylostoma caninum	>10 kDa 0.2 µm filtered	Mouse chemical colitis models	Induction of modified Th2 response	[256] [5]
Trichinella spiralis	0.45 µm filtered	Mouse chemical colitis model	Induction of modified Th2 response	[314]
Brugia malayi	0.45 µm filtered	*In vitro* stimulation of human PBMC	↑ IL-6 ↑ IL-8 ↑ VEGF-A	[315]
	0.2 µm filtered	*In vitro* suppression of murine D10.G4	Suppression of Th2 cells ↑ IL-4	[311]
Toxocara canis	0.2 µm filtered	*In vitro* suppression of murine D10.G4	Suppression of Th2 cells ↑ IL-4	[311]
Taenia sp.	>50 kDa	*In vitro* stimulation of human PBMC	Tolerogenic phenotype Reduction of pro-inflammatory cytokines Modulation of CLR	[316]

Table 2.5. Variability in preparation techniques and ICH screening approaches for different helminth excretory/secretory (ES) products with immunomodulatory properties.

ES = Excretory/Secretory; BMDC = Bone marrow derived cells; IPEC-1 = piglet intestinal cell line; PBMC = peripheral blood mononuclear cell; CLR = C-Type Lectin Receptor

2.13 Successful helminths induce a 'modified Th2' response in the host through ICH.

It is recognised that parasitic helminths are often expelled from the host via a T helper type 2 (Th2) response, typified by elevated secretion of IL-3, IL-4, IL-5, and IL-13, Immunoglobulin E (IgE), eosinophilia, alternatively activated macrophages and mastocytosis [317, 318]. IL-4

and IL-5 produced by Th2 cells are implicated in cross talk with B cells and eosinophils and, ultimately, degranulation of mast cells, basophils and eosinophils. It is this cascade that is thought to be responsible for parasite expulsion [319]. This can be seen in the whipworm, *Trichuris trichiura* where the immune response to infection results in chronic diarrhoea and protein losing enteropathy in the form of Trichuris Dysentery Syndrome. This is recognised as an inflammatory response with the involvement of the innate immune system [320] and is well defined in mice infected with the related *Trichuris muris*, where the bias towards a Th2 response results in expulsion of the parasite [321-324].

The clear majority of human helminth infections are characterised by a partial activation of both the adaptive and innate arms of the immune system termed a 'modified Th2" response. While helminth parasites clearly cause numerous neglected tropical diseases, with soil transmitted helminths (STH) estimated to affect over 2.2 billion people [48, 49], their effect is considered more in terms of morbidity than mortality [48]. In this context, host and parasite appear to have struck a balance between persistent infections over the lifespan of the host, but most infected individuals harbour low to moderate worm burdens, thereby preventing uncontrollable inflammatory responses and death of the host.

A successful parasite is benchmarked by its ability to actively skew its host's immune system to prevent elimination and development of immunity [50]. This immune skewing results in the induction of a 'modified Th2' response [51] characterised by expansion of Th2 cells, alternatively activated macrophages, inducible regulatory T cells (Treg) expressing Foxp3, increased levels of IL-10, and production of polyclonal IgE by B cells [55], and consequent suppression of the Th1/Th17 axis. Classical Th1/17 inflammatory responses can damage worms, but at a substantial cost in collateral damage to host tissues [325]. Thus, in evolutionary terms, Th2 immunity is thought to have arisen from tissue repair mechanisms in response to tissue damage, with these responses isolating and encapsulating helminths through fibrotic processes while simultaneously resolving localised damage [325-327]. Indeed, parasitic helminths are one of the most adept infectious agents at driving regulatory responses that prevent the development of sterilising protective immunity. Most relevant from the perspective of this article is the added benefit of helminth-driven immunoregulation in suppression of inappropriate immune responses to self and otherwise innocuous antigens [55]. With the exception of the liver fluke *Opisthorchis viverrini,* which is

associated with inflammatory changes in the liver [313, 328], ES products from the majority of helminths investigated induce a tolerogenic immune phenotype characterised by reduced production of pro-inflammatory cytokines. The ability of a helminth (or its ES products) to induce a regulatory response rather than an overt inflammatory response is clearly critical when searching for ICH.

2.14 The complex nature of ICH

ES products consist of a complex mixture of compounds of diverse molecular nature and function. Hewitson and colleagues mined various helminth ES proteomes from which they identified nine major ICH motifs on the basis of both experimental evidence from functional studies and genomic analyses to identify homologues of known immunomodulators [329]. The complexity of ES proteins and limitations in available quantities for separation studies has meant that current ICH discovery efforts have often focused on the most predominant ES components. For example, ES products of the canine hookworm *Ancylostoma caninum* protect mice against chemical colitis [5, 256]. A proteomic analysis of *A. caninum* ES products identified 105 proteins [161], many of which have predicted immunomodulatory properties based on sequence similarity to mammalian homologues including C-type lectins, galectins and cytokines. Crude *A. caninum* ES products when injected into mice induce a polarised Th2 response [256]. In contrast however, the most abundant protein in *A. caninum* ES products shares sequence identity with the human Tissue Inhibitor of Metalloprotease (TIMP) family, and has been shown to suppresses dendritic cell activation and drive production of regulatory T cells and regulatory cytokines [246] rather than polarised Th2 responses. On the other hand, ES products of the filarial nematode *Acanthocheilonema viteae* are dominated by the phosphorylcholine (PC)-containing immunoregulatory glycoprotein ES-62 [330]. ES products from other parasitic nematodes display similar molecular complexities and Th2-inducing capacities, but little information has been obtained on the individual ICH and how these contribute to defined immunoregulatory pathways. For example, *Heligmosomoides polygyrus* ES products contain eight protein families with predicted ICH activity [52] and *Brugia malayi* secretes at least 228 proteins including the immunomodulatory galectins and proteins modified with PC [206, 207]. Some parasitic trematodes also display similarly complex ES products - *Schistosoma mansoni* secretes approximately 188 detectable proteins during its life cycle, including at least four

that are known to be associated with immunomodulation [331]. While proteomics has served to highlight potential ICH within ES products, *in vitro* and *in vivo* studies are required to unequivocally attribute this function to crude mixtures and defined purified proteins (Table 2). Such studies provide the requisite information on the therapeutic potential of an ICH for clinical development and pique the interest of commercial partners [332] as they display specific pharmacology relevant to therapeutic discovery.

2.15 ICH components are pleiotropic

ICH of helminths are increasingly found to have multiple host targets and can modulate the mammalian immune system in many distinct ways [329]. Identification and characterisation of individual ICH is facilitating the recognition of motifs that have been adopted by a number of helminths to modulate the immune system, including defined post-translational modifications such as PC [333, 334]. These molecules are generally large and antigenic, but the bioactive component is often substantially smaller and therefore of greater interest in terms of drug development [332]. One of the best-studied ICH is the 62 kDa ES protein from *A. viteae*, ES-62. The addition of PC via an *N*-type glycan bond results in both the induction of an anti-inflammatory phenotype in mice [335] which is entirely dependent on the presence of PC. Indeed, the same anti-inflammatory effect was obtained when PC was fused to bovine serum albumin as a carrier protein [336]. The cellular targets of ES-62 are very broad, and include T cells, B cells, macrophages, mast cells and dendritic cells [329]. ES-62 (via its PC epitope) has shown therapeutic potential for the treatment of inflammatory diseases [333] and protects against inflammation in mouse models of asthma [337] and arthritis [338]. Due to the antigenicity of the ES-62 protein its development as a therapeutic drug is not possible, but small molecule derivatives obtained from the PC moiety of ES-62 have been synthesised and show promise in the treatment of collagen induced arthritis [339]. The PC motif is not well represented in helminth ES products – it has been identified in *Ascaris suum*, where glycosphingolipids have both PC- and non PC-dependent immunomodulatory activity [340], but given its potency, the scarcity of PC in other helminth ES products is unexpected, and reflects the many distinct ICH and their often divergent mechanisms used to manipulate the immune system.

In addition to protein and phospholipid ICH, helminths produce unique glycans with pleiotropic immunomodulatory properties [206, 334]. Glycans from helminths have been demonstrated to induce both C-type lectin receptor (CLR) signalling and cross talk with Toll-like receptors (TLR) [334, 341]. Glycan moieties of ICH have been described with both protective and non-protective properties in inflammatory disease models, and the ability to recognise relevant anti-inflammatory motifs is key to unravelling their therapeutic value [334, 342, 343]. This is perhaps best reflected in the study of the glycans of the schistosomes. Much of the antibody response to schistosomes during infection of primates is targeted towards glycans [344]. These glycans have been implicated in "gimmickry" to evade expulsion [334] and increase ova survival [344]. Schistosome glycans containing the motifs LacdiNAc- and LacNAc have been shown to promote the formation of pro-inflammatory granulomas [345], while the glycan motifs IPSE/α1 and Kappa-5 induce Th2 biasing in dendritic cells (DCs) [346]. The ability of helminth derived glycans bearing a Lacto-N-fucopentaose III structure to improve glucose tolerance and reduce hepatosteatosis (fatty liver) in mice fed on a high fat, high carbohydrate diet has led to the suggestion that this glycan may provide a novel approach to treat metabolic disease [347].

2.16 ICH bioactivity cannot always be predicted by current bioinformatics modelling

The limitations of bioinformatics become apparent when considered in terms of predicting ICH. Nucleic acid and protein sequence databases can be used to predict the secretome of an organism [348, 349], yet despite recent progress in the sequencing of numerous parasitic helminth genomes and transcriptomes, the ability to predict ICH bioactivity has been limited. In the case of ES-62, filarial cystatins (see below) and hookworm TIMPS, ICH activity has been identified from ES products through bioactivity screening rather than bioinformatics modelling.

When ES-62 was first identified from *A. vitae* it was initially speculated from the bioinformatics data available that the protein had homology to aminopeptidases [350]; however the protein displayed only weak aminopeptidase activity [350], and instead its immunomodulatory properties were assigned to the PC moiety [351]. Where PC is represented in helminths [352, 353] there are indications that they are highly conserved

structures [354]. Current bioinformatics tools focusing on protein and nucleic acid sequence identities and predicted folds would have not revealed the immunomodulatory properties of ES-62, a and molecule that is currently being targeted for the development of numerous inflammatory indications [337, 338].

The extensive body of work on helminth cystatins shows that homology as revealed by bioinformatics may not reflect the ICH properties of these molecules. The cystatins are reversible cysteine protease inhibitors with ubiquitous distribution [355, 356]. While proteomic analyses of ES products reveals an abundance of cystatins and cystatin-like proteins [207, 355] the widespread occurrence of the cysteine proteases and their cystatin and other inhibitors [356] would have made it easy to dismiss their roles in helminth-driven immunoregulation. Indeed early publications revealed a role for protozoan parasite cystatins in driving inflammatory responses by promoting nitric oxide (NO) production in macrophages in a protease inhibitor-independent manner [357]. Shortly afterwards however, the same researchers described cystatins from filarial nematodes that down regulated T cell proliferation and increased the production of IL-10 [358, 359]. Subsequently, modified filarial cystatin has been used in mouse models of allergy with promising results [304, 360].

The genome of the human hookworm *N. americanus* [60, 175] was recently published, and revealed a number of genes encoding for proteins with sequence similarity to TIMPs. However it was only through *in vitro* studies and detailed modelling that the apparent lack of TIMP-like activity of *Ac*-TMP-1 became apparent [58], suggesting that this protein family had developed an alternative mechanism of suppressing inflammation [246] that was independent of metalloprotease inhibition. This combined with lack of functional data and the diversity of helminth ICH can limit the interpretation of analysis and this limits the ability to detect and predict ICH motifs.

The ability of bioinformatics to identify potential ICH is highlighted by the discovery of *Stichodactyla helianthus toxin* (ShK)-related peptides from parasitic nematodes. This peptide was first identified in sea anemones as an inhibitor of voltage gated Kv1.3 K^+ channels of effector T cells and a therapeutic target for the treatment of autoimmune disease [361]; a structural analogue of ShK-186 [362] is currently in phase 1 clinical trials for the treatment

of a number of autoimmune diseases (http://www.kinetabio.com/newsArchive.html). Using bioinformatics-based approaches, homologues that suppressed T cell proliferation and IFN-γ production *in vitro* were identified in tissue-dwelling (*Brugia malayi*) and gastrointestinal (*Ancylostoma sp.*) nematodes [257]. The ShK domain has been described in ES products of other helminths including *H. polygyrus* [363] and *T. canis* [364], however their role in host-parasite interactions has received little attention.

Increasingly, post-translational modifications such as PC and various glycans are being recognised as the active components of many ICH [329, 334]. These unique molecules cannot be readily predicted by bioinformatics approaches, and it is only through direct experimental investigation that these molecules reveal their ICH properties [365]. The potential power of bioinformatics must not be discounted; the ability to map motifs associated with ICH will be a valuable tool to identify convergent strategies of parasitic helminths.

2.17 Small organic molecule ICH – unexplored pharmacophores?

Most of the current literature on ICH has focused on proteins. Pursuit of low molecular weight ICH is however worthy of attention due to their improved drug development properties, such as Lipinski's rule of five [24, 332]. Additionally, the use of peptides as therapeutics provides the means to generate highly targeted, complex molecules that negate some of the challenges of protein-based therapeutics, including delivery systems and potential antigenicity of large proteins [366].

Peptide ICH have received little attention other than recent work on helminth defensin-like molecules [367, 368], a family of fluke peptides that do not possess anti-microbial properties like their mammalian counterparts but instead display a range of immunoregulatory roles, primarily through interruption of macrophage antigen processing pathways [369]. Genome sequences are available for many of the parasitic helminths but traditional open reading frame (ORF) prediction programs often discount short ORFs that correspond to small peptides, so tailored approaches are required to mine genomes for small peptides.

Small organic molecules from helminths have received even less attention as ICH, despite their likely presence in ES products [329]. The study of small molecules shifts the experimental discovery paradigm from proteomic and genomic strategies to that of metabolomics. For instance, the eicosanoids are small oxylipic molecules that are recognised ICH with pleiotropic behaviour and are produced by a wide variety of organisms [212]. The parasitic helminths are known to produce prostaglandin and leukotriene eicosanoids [212, 370, 371]. For example, the cercariae of *Schistosoma mansoni* utilise the linoleic acid present in the host epidermis to produce a prostaglandin, PGE_2, implicated in IL-10 dependent down regulation of host immune responses required for successful migration of the parasite [372]. These helminth-derived molecules have not (to our knowledge) been screened in animal models of inflammatory diseases to assess their therapeutic potential. Indeed there has been surprisingly little research into the low molecular weight ES component of parasitic helminths despite promising evidence on their therapeutic properties [373].

Also worthy of further attention is the ascarosides, a class of small molecules that have been well characterised in *C. elegans* [374] and are present in other helminths [375]. Ascarosides are synthesised by peroxisomal β-oxidation of long chain fatty acids and amino acid catabolism [376], and as a result the ascaroside distribution pattern provides information about the life history and the metabolic status of the worm [374, 375]. Ascarosides are chemically stable and display diverse bioactivity at femtomolar concentrations [374] as dauer pheromones, male-specific attractants and aggregation signals [377, 378]. They are highly species-specific signalling molecules [375] recognised by cyclic guanosine monophosphate membrane receptors (cGMPR) on the helminth's surface and upstream of TFG-β signalling pathways [376, 379]. While similar compounds are present in the ES products of parasitic nematodes [375] their role in host-parasite biology is yet to be fully explored.

Finally, the immunoregulatory role of short chain fatty acids (SCFAs) from commensal microbes has become apparent [380], yet their presence in parasitic helminths has received little attention. Even though parasitic nematodes secrete some of the key immunoregulatory SCFAs including propionate and butyrate [381-383], their contribution to host-parasite immunobiology has not been addressed in depth.

While the area of small molecule ICH is worthy of further exploration, we offer a word of caution in terms of selection of *in vitro* culture media used for helminths. Small molecule profiles are profoundly affected by the metabolic status of the organism [374, 375]. Proteomic analyses typically focus on proteins greater than 10 kDa in mass [161, 307, 308, 312, 316]. The analysis of smaller molecules in ES products can be confounded by protein breakdown products and media components including amino acids that have immunomodulatory properties in their own right. For example, the commonly used tissue/helminth culture medium RPMI-1640 contains choline [384], glutamine and arginine [385], thiamine [82] and tryptophan [386], all of which have been shown to have anti-inflammatory properties, while glucose has pro-inflammatory properties [387]. Indeed glutamine is administered to critically ill patients, where it reduces mortality rates [388] and lessens inflammation post-chemotherapy [389] via its non-nutritive roles in modulation of the immune system [390]. This complicates the use of ES products from parasitic helminths that contain media components, particularly if a concentration/precipitation step has been applied.

2.18 Conclusion

Many helminths and their ES products are viewed as a valuable source of potential therapeutic moieties [117]. Parasitic helminths produce a large repertoire of ES proteins, a number of which have been characterised and display immunomodulatory potential. To date much of the work on ICH as focussed on proteins with only preliminary investigation into the therapeutic potential of low molecular weight peptides and small organic molecules [373]. Indications from free-living helminths such as *C. elegans* suggest a diversity and complexity in bioactive molecules that play roles in both helminth-host immunobiology and as novel therapeutics for inflammatory and other non-infectious diseases. Despite the difficulties of developing proteins as therapeutic agents there is growing interest from commercial partners in the development of these compounds into therapeutics [332]. This is mainly because proteins are highly specific, targeted and proven through long association to be well tolerated. They also address the very real need for curative treatment options for a wide range of inflammatory, autoimmune and allergic diseases. Drug discovery guided by millennia of host-parasite co-evolution is logical, and immunoparasitologists are now at the forefront of this exciting era where the need for out-of-the-box or unconventional

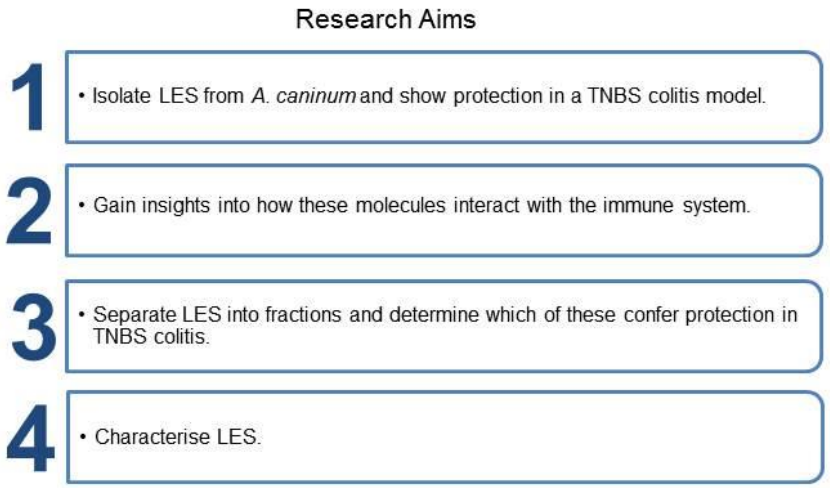
approaches to developing drugs to treat the epidemic of inflammatory diseases is urgently needed. Parasitic helminths are masters at preventing inflammation, and ICH-derived therapeutics offer truly disease-modifying approaches, rather than the focus of current biologics, many of which are designed to “mop up” the damage rather than acting upstream to reset the effector: regulatory balance. We believe that ICH-derived therapeutics have a place in modern medicine and will be available in the clinic in the not too distant future.

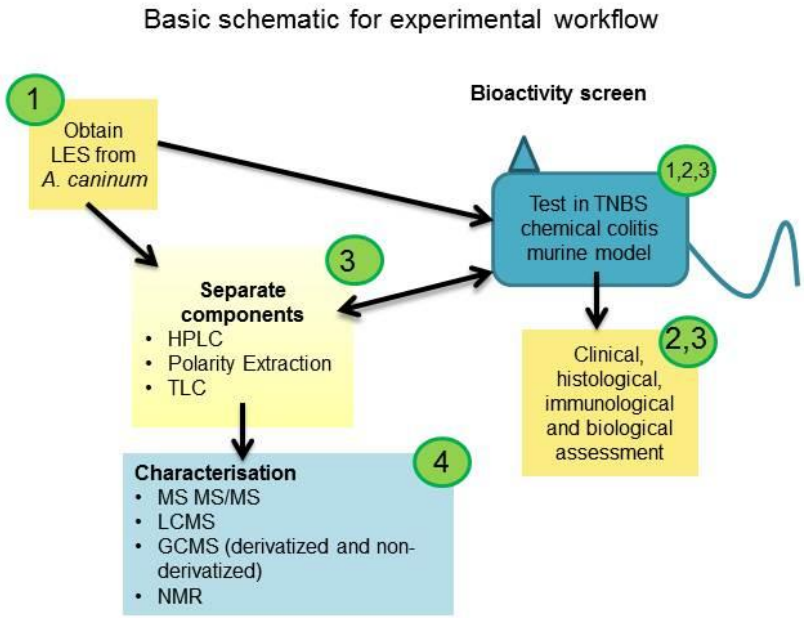
Chapter 3

Materials and Methods

Preamble

This chapter outlines the experimental procedures and protocols utilised during this thesis to achieve the research aims. This chapter also addresses the experimental plan, statistical and bioinformatics workflows for the project. Research aims are;





Above is a schematic of the experimental workflow; the numbers listed are the research aims that are addressed at each stage of the project.

The following procedures are listed in the approximate order in which they appear in the research aims of the project. A more detailed research plan with rationale is provided in Chapter 2.

3.1 RESEARCH AIM 1: Isolate LES from *A. caninum* and show protection in a Trinitrobenzene Sulphonic Acid (TNBS) colitis model.

This section covers

- Isolation of *A. caninum* and culture.
- Preparation of ES and LES – the testing of protein concentration (BCA) and endotoxin contamination levels (LAL).
- Sample preparation methods – lyophilisation, preparation for IP injection.
- Bioactivity assay – Acute chemical colitis induced by TNBS
- Histology – H&E stain and PAS stain

3.1.1 Procedure: Preparation of whole Excretory/Secretory component from *A. caninum*.

Scope: This procedure covers the harvesting of intestines from the dog to the preparation of ES material from parasites for transport. It is assumed that the investigator can identify ova from and determine the species of adult hookworms. This method is adapted from Mulvenna *et al.* [161]

Safety/Notes: Dog parasites can cause zoonotic infections in humans. Full PPE including gown, gloves and surgical mask is recommended. In addition plastic apron and sleeve protectors are advised. Appropriate safety procedures should be adopted using when using scalpel blades and appropriate disposal of sharps is required.

Materials/Instrumentation:

Equipment Requirements:

- CO_2 (5%) Incubator at 37°C
- Waterbath (37°C) for warming Substrates
- Laminar Flow Cabinet
- Centrifuge – 50 ml tubes/1000 *g*/10 minutes/4°C
- Large plastic tray
- Curved surgical scissors
- Watchmaker tweezers
- Pipette boy

• Scalpel

Consumables and reagents:

• Petri dishes (25 mL capacity)

• 50 mL Falcon tubes

• Pipette boy 25 mL tips

• 5x Substrate – Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (Gibco, Life Technologies) + 5% antimycotic/antibiotic (Gibco, Life Technologies) + 1% Glutamax (Gibco, Life Technologies). i.e. 500 mL DPBS + 25 ml anti/anti + 5 ml Glutamax

• 2x Substrate – Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (Gibco, Life Technologies) + 2% antimycotic/antibiotic + 1% Glutamax. i.e. 500 mL DPBS + 10 ml anti/anti + 5 ml Glutamax

Substrate is prepared aseptically and can be stored at 4°C prior to use. Warm Substrate in a 37°C waterbath before to use (for every 50 hookworms approximately 75 ml of 2x Substrate will be required for a 3-day culture).

Method:

Collection of Intestines

Hookworms are obtained from the small intestine of dog cadavers within 180 minutes of lethal injection with sodium-pentobarbital. Intestines should be harvested from freshly euthanized animals that are suspected of hookworm infestation (this can be confirmed by faecal examination for hookworm eggs). Hookworms will detach from the gut as the tissue cools that will make it difficult to identify worms. Processing intestines quickly is essential to maintain integrity of the parasite.

Removal of the small intestine from dogs

1. Using a scalpel make a small transverse cut on the lower abdomen of the animal. Cut through the dermis and the abdominal lining carefully to expose a small amount of the small intestine.

2. Using a finger, loop under the small intestine and gently pull the intestine through this hole. Locate the junction to either the large intestine or stomach. Cut the intestine at this point and gently pull the small intestine out, easing it away from the mesenteric membranes until the other end is reached. Tie each end of the intestine to

prevent content loss. Place in a container for transport to the laboratory. Dispose of the animal as approved.

Hookworm removal and processing

1. Process intestines over a large plastic tray.
2. Using curved surgical scissors cut down the length of the intestine to expose the inner surface.
3. Using clockmaker tweezers gently pick the hookworms from the intestine and transfer to 5x substrate (minimum 25mL). Avoid any faecal material and intestinal tissue.
4. Centre the worms by swirling the container for several seconds. While the worms are centred use a pipette boy with a 25 ml tip to remove as much of the liquid as possible without sucking up the worms. Discard the Substrate.
5. Quickly replace media with warm 5x Substrate to the container.
6. Repeat steps 4 to 5 at least 4 times (more if needed) to remove any visible intestinal matter. Centre the worms then using a new 25 ml tip aspirate the worms ensuring to remove as little liquid as possible. Place approximately **50** worms per Petri dish.
7. Transfer worms to a petri dish and add 25 mL of 5x Substrate. Incubate for 2 hours at 37°C in a CO_2 incubator.

ES Production from hookworms (all work to be carried out in laminar flow cabinet)

1. During the 2-hour incubation period clean all equipment and wipe down the laminar flow hood with ethanol. *This incubation period is important as it allows the 5x anti/anti component to neutralise the bacteria present and the hookworms to purge their gut contents.*
2. Remove petri dishes from incubator. Centre the worms by swirling petri dish then using a new 25 ml tip suck up all the worms making sure that you aspirate as little media as possible. Transfer to a new petri dish containing 2x Substrate.
3. Retain the 5x Substrate and process as per steps 7-8. Label these tubes clearly as **5x ES** with the date. Store at -20°C or -80°C until needed.
4. Wash the hookworms 3 times as previously but with 2x Substrate.

5. Incubate worms in approximately 25 ml of 2x Substrate for 16 hours in a 37°C CO_2 incubator (This is **Day 1** ES collection material).

6. After 16 hours incubation collect Substrate (contains ES proteins) using a 25 ml tip and transfer to a 50 ml falcon tube (more than one petri dish can be pooled into a falcon tube).

7. Centrifuge at 1000 *g* for 10 minutes at 4°C to pellet the hookworm eggs. These pellets are retained, and an egg shed is estimated. A live to dead ratio can also be estimated by staining eggs with Lugol's iodine.

8. Transfer the supernatant to a new tube – you can pool but only up to 40 ml (to allow for expansion in the freezer). *Label tube clearly with day of ES collection and date.* Store at -20°C or -80°C until needed.

9. Repeat steps 4 to 7 for **Day 2** and **Day 3** collections. **Remove dead worms with tweezers between each incubation** (these worms will show signs of disintegration or will not be moving in the Substrate – nudge motionless worms with the tweezers- they should move if alive). Store at -20°C or -80°C until needed. This material is now ready for transport if required.

Quality Control: The identity of hookworms needs to be confirmed visually (three pairs of teeth in the buccal capsule) using a dissecting microscope to ensure that *A. caninum* is being collected for study.

3.1.2 Procedure: Preparation of *A. caninum* Excretory/Secretory component (AcES) and low molecular weight AcES (LES) for analysis.

Scope: This procedure covers the thawing of whole ES material from *A. caninum* and the separation of the low molecular weight component and >10 kDa protein component for characterisation and analysis. This method is adapted from Mulvenna *et al.* [161]

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate safety procedures should be adopted when using scalpel blades and appropriate disposal of sharps is required.

Materials/Instrumentation:

Equipment Requirements:

- Laminar Flow Cabinet
- Centrifuge – 50 ml tubes/3000 *g*/20 minutes/4°C

Consumables and reagents:

- 50 mL Falcon tubes
- DPBS
- 10 kDa spin column

Method:

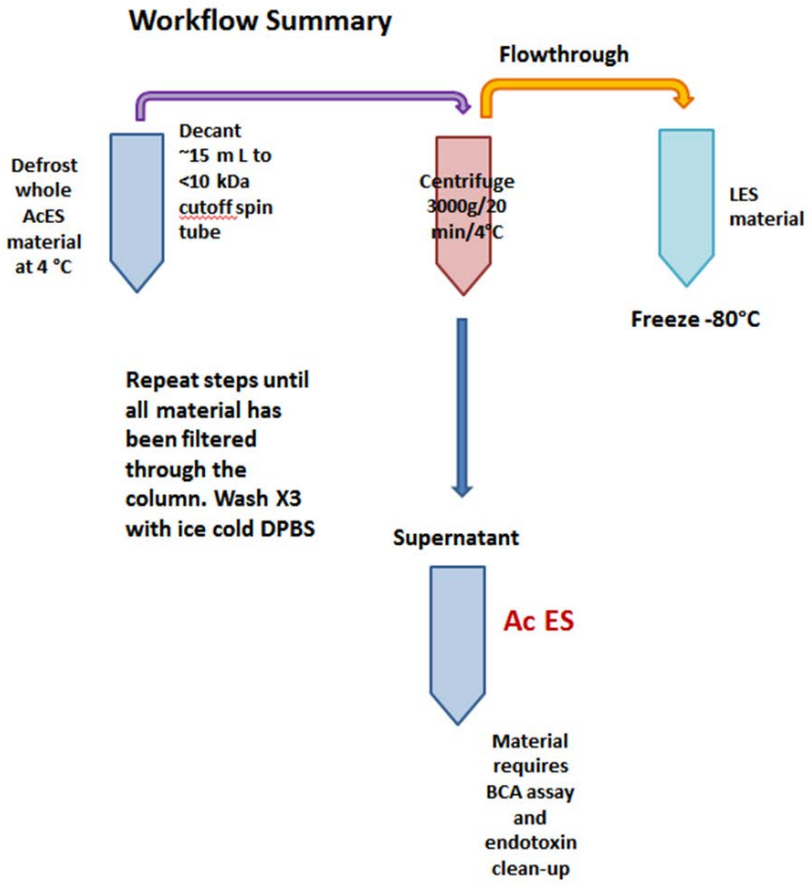


Figure 3.1 Summary of experimental workflow for ***A.*** *caninum* Excretory/Secretory component (AcES) and low molecular weight AcES (LES) preparation.

Concentration of AcES and LES

1. Defrost whole ES material at 4°C and keep tubes on ice throughout the procedure. Open tubes and decant liquid in the laminar flow hood to minimise risk of contamination.
2. Decant approximately 15 mL of whole ES into the 10 kDa cut off spin column.
3. Centrifuge at 3000 *g* for 20 minutes at 4°C.
4. Transfer the flowthrough to an appropriately labelled 50 mL falcon tube. Top up the 10 kDa column with the whole ES material. Repeat step 2 until all material has been loaded through the column.
5. Once all the AcES material is loaded onto the column start topping up with cold DPBS.
6. Do at least three washes with the DPBS.
7. Concentrate the AcES to approximately one millilitre in each spin column. Rinse the empty spinoff tube with 100µL of cold DPBS. Carefully rinse and remove as much ES material as possible. Transfer this material to a 1.5 mL microfuge tube (Microfuge) and proceed to the endotoxin clean up procedure.
8. The flow-through material is considered as the LES and is now suitable for characterisation. Material can be stored at -80°C.

3.1.2.1 Procedure: Removal of endotoxin from AcES

Scope: This procedure covers the removal of endotoxin from AcES for characterisation and analysis. This method is adapted from Aida and Past [391].

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate safety procedures should be adopted using when using scalpel blades and appropriate disposal of sharps is required.

Materials/Instrumentation:

Equipment Requirements:

- Laminar Flow Cabinet
- Microfuge Centrifuge – suitable for 1.5 mL Microfuge tubes

- Waterbath 37°C
- Rotary mixer at 4°C

Consumables and reagents:

- Microfuge tubes (1.5 mL)
- Triton X114 (Sigma)

Method:

1. In a laminar flow cabinet transfer approximately 500µL of AcES to a Microfuge tube.
2. To this tube add 1 % Triton X114 to the AcES (µL). Open tubes and decant liquid in the laminar flow hood to minimise risk of contamination.
3. Place tubes on a rotary mixer and mix on medium speed for at least 30 minutes – overnight. This causes the Triton X114 to bind to the endotoxin.
4. Heat tubes to 37°C for 10 minutes. This causes the Triton X114 to form micelles and trap the endotoxin.
5. Centrifuge the Microfuge tubes at 17 500 g for 15 minutes at room temperature. The liquid will separate into two layers – the bottom layer contains the endotoxin.
6. In a laminar flow hood decant the top layer and add to a fresh tube, then repeat step 2.
7. Warm the supernatant in a waterbath at 37°C for 5 minutes and centrifuge again for 15 minutes.
8. Transfer the top layer (ensuring to not transfer any of the bottom layer) into a new tube to ensure the sample is completely free of Triton X114.
9. Assay AcES material for endotoxin levels using the LAL method and protein concentration using the BCA method.

3.1.2.2 Procedure: endotoxin concentration determination using the Limulus Amebocyte Lysate (LAL) assay.

Scope: This procedure covers the preparation of sample to the assay and calculation of bacterial contamination in the form of lipopolysaccharide (LPS) concentration of samples. Samples that contain excessive LPS quantities cannot be used in the TNBS

mouse colitis model and should be processed through the endotoxin removal method until acceptable levels are obtained.

Note: The LPS molecule is very large and is contained in the ES fraction. LES will not contain measurable quantities of LPS

Safety/Notes: Full PPE including gown and gloves is recommended.

Materials/Instrumentation:

Equipment Requirements:

- Automatic pipettes
- Incubator 37°C (optional)
- Automated plate reader – Polarstar Omega (BMG Labtech)

Consumables and reagents:

- Limulus Amebocyte Lysate (LAL) kit (Lonza-Pharma Biotech)
- Microfuge tubes (for dilutions)
- Buffer for dilutions (match to that of the sample)
- 96 well microplate

Method:

1. Method is carried out as per the assay kit insert and is briefly summarised below.
2. Prepare standards as indicated in insert using compatible buffer. Samples should be serially diluted for the best determination of concentration.
3. Add 50µL of sample or standard to each well followed by 50µL of sterile DI water
4. Add 50µL of LAL reagent to each well and mix the plate thoroughly.
5. Cover the plate and incubate at 37°C for 10 minutes.
6. Add 100µL of Substrate to each well and mix. Incubate plate for a further 6 minutes at 37°C.
7. Add 100µL of stop solution to each well and mix.

8. Read plate at 405-410 nm and determine the concentration of unknown samples using the established standard curve. Use DI water as a sample blank.

3.1.2.3 Procedure: Protein concentration determination using the Pierce BCA protein assay kit.

Scope: This procedure covers the preparation of sample to the assay and calculation of protein concentrations.

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate safety procedures should be adopted using when using scalpel blades and appropriate disposal of sharps is required.

Materials/Instrumentation:

Equipment Requirements:

- Automatic pipettes
- Incubator 37°C (optional)
- Automated plate reader

Consumables and reagents:

- Pierce BCA Protein Assay Kit (Thermo Scientific)
- Microfuge tubes (for dilutions)
- Buffer for dilutions (match to that of the sample)
- 96 well microplate

Method:

1. Method is carried out as per the assay kit insert and is briefly summarised below.
2. Albumin standards are prepared in the following concentrations; 2000, 1500, 1000, 750, 500, 250, 125, 125, 25 and 0µg/mL using kit as guide and matching compatible buffer.
3. Make up working reagent allowing 200µL per sample (50:1, reagent A:B)
4. Add 25µL of sample or standard (to be run in duplicate) to each well.

5. Add 200µL of the working reagent to each well and mix the plate thoroughly on a shaker for 30 seconds.

6. Cover the plate and incubate at 37°C for 30 minutes (room temperature for 2 hours).

7. Cool plate to room temperature if incubated at 37°C.

8. Read plate at 562 nm and determine the concentration of unknown samples using the established standard curve.

3.1.3 Sample Processing

3.1.3.1 Procedure: Sample lyophilisation (freeze drying) or concentration

Scope: This procedure describes the various methods used to concentrate samples through lyophilisation/evaporation.

Safety/Notes: Full PPE including gown and gloves is recommended. Safety glasses must be worn around lyophilisation equipment. The use of dry ice should be carried out in a ventilated space and solvents should be disposed of appropriately.

Materials/Instrumentation:

Equipment Requirements:

- Fume hood.
- Freeze dryer – Christ Alpha 1-2 LD Plus (John Morris Scientific)
- Round bottom flasks compatible with freeze dryer unit. Select flask that is approximately four times the volume being dried.

Consumables and reagents:

- 50 mL Falcon tubes
- Microfuge tubes
- Glass vials
- Nitrogen gas (BOC gases)
- Dry ice (commercially available from Collins Ice, Cairns)
- Ethanol or Acetone (reagent grade)

• Parafilm

Method:

Large volume method – lyophilisation method A

1. To be done in a fume hood. To a suitable container add approximately one cup of dry ice and either ethanol or acetone. This mixture can descend to temperatures as low as -80°C – appropriate precautions are to be taken.
2. Transfer liquid to a round bottom flask, carefully avoiding getting the ground glass neck wet. Place an adaptor arm on the flask and parafilm junction securely.
3. Using a rotating motion move flask in bath ensuring that the solution is frozen evenly over the interior surface of the flask. Make sure that the liquid has completely frozen before loading onto the freeze dryer.
4. Once the sample is completely frozen load onto the freeze dryer.
5. Drying can occur overnight or over the course of a number of days. The sample needs to be monitored closely. If the sample thaws during the process it must be removed, refrozen and placed back on the dryer.
6. Once completely dry carefully disengage the vacuum to minimise sample loss and remove flask. Dried sample is now available for processing as required.

Large volume method – lyophilisation method B

7. In some cases, samples will be provided frozen in 50 mL falcon tubes and it will be impractical to thaw and transfer these samples. In these cases, a large jar suitable for containing several tubes at once can be used. Remove the lid from each falcon tube and cover with parafilm. Poke several holes through parafilm. Work quickly to ensure that the sample remains frozen.
8. Once prepared samples can be loaded as per step 4 of the method.
9. When samples are dry they can be removed and processed as required.

Large volume method – Rotary Evaporator method C

1. Add ice to the condenser and turn on the water bath to bring up to temperature.

2. Turn on water to vacuum.

3. Load sample and start spinning hold until vacuum is reduced to avoid sample loss lower into the water.

4. Set mode to pump down (select using the knob dis-engage the lock and press down) at the first bubble – stop the vacuum.

5. Change mode to auto vacuum then watch to achieve volume required. Raise flask out of bath.

6. Vent while holding flask.

7. Shutdown by turning off the rotation, heater to water bath. Vent vacuum and power down both units. Discard ice; when bath is cool, empty and dry.

Small volume method – lyophilisation method A

1. When small volumes of sample need to be evaporated they can be processed using the Speed vac. Samples in Microfuge tubes - in either liquid or frozen form - are loaded into the rotor of the Speed vac (balance rotor if possible).

2. Press lid down to engage lock and start spinning process.

3. When lock is engaged the vacuum will start and samples will lyophilize. Check regularly

This method is not suitable for highly volatile or labile compounds and method B is more suitable.

Small volume method – lyophilisation method B

1. When small volumes of samples contain volatile or labile components it may be more suitable to dry samples under nitrogen.

2. Using bottled nitrogen gas and in a fume hood direct a small stream of nitrogen into the container that contains the sample. Evaporate to dryness.

3. Depending on the amount of sample this may take a few minutes, but the liquid should evaporate, concentrating the sample – this can be to completely dry the sample or just to concentrate it.

3.1.3.2 Procedure: Preparation of biological samples for intraperitoneal injections.

Scope: This procedure covers the preparation of biological samples for animal injection. This procedure is covered by JCU Ethics A#1848, A#2190 and A#2012.

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate safety procedures should be adopted when using scalpel blades and syringes and appropriate disposal of sharps is required.

The administration of anaesthetic as described in this procedure requires a current DS4-DS8 approval from the Queensland Government. A copy of this approval is held by the facility.

This procedure also requires induction into the Specific Pathogen Free (SPF) facility, instruction in the correct handling of small mammals, the record of which will be kept by the manager of the facility.

Adherence to SPF facility safety and requirements is mandatory.

Materials/Instrumentation:

Equipment Requirements:

- Laminar Flow Cabinet

Consumables and reagents:

- Microfuge tubes
- 0.22 µm filter cartridges - Millex syringe filter unit 33 mm/4 mm (Merck Millipore Ltd)
- Sterile syringes, 19 G, 21 G and 23G needles (Termo)
- Insulin syringes (Termo)
- DPBS
- Dimethyl sulfoxide (DMSO)
- Ketamil - Ketamine (as Hydrochloride) 100 mg/mL (Ilium)
- Xylazil - (2-(2,6 Xylidine)- 5,6 dihydro-4H-1,3-thiazine hydrochloride) 100 mg (10% w/v) (Ilium)

Method:

Experimental

General Notes – The total volume that can be administered at any one time is 200 µL to each mouse. All samples must be prepared aseptically in a laminar flow as bacterial/LPS contamination will alter assay.

1. **Preparation of material for screening –** All mouse injections need to be prepared aseptically in a laminar flow cabinet. Sample is to be made up to the correct concentration in a 200µL volume. Using sterile diluent (usually sterile PBS) take up enough to allow for dead volume in the tube (usually done by calculating n+1). Material that cannot be heat sterilised is to be filtered through a 0.22 µm filter and stored at 4°C until use. In some cases, it is necessary to use DMSO to solubilize samples. If DMSO is used, then a control group using diluent sample only is required in the bioactivity assay.
2. **Preparation of anaesthetic** – To administer these drugs current DS4-DS8 approval from the Queensland Government is required to have access to and administer these drugs. Drugs are to be stored in a secure location and usage is to be recorded in drug usage logs. Anaesthetic is to be made on an adhoc basis only. Anaesthetic is prepared aseptically to a concentration of 50 mg/kg of Ketamil and 5 mg/kg of Xylazil is prepared in sterile PBS at a rate of 200µL per mouse (allow for dead volume in requirement volume). Prepared material is to be stored at 4°C until use.

3.1.4 Biological Screening for anti-inflammatory properties

3.1.4.1 Procedure: Murine Trinitrobenzene sulfonic acid (TNBS) colitis method

Scope: This procedure covers the preparation of TNBS, administration and monitoring of mice during this experiment. JCU Ethics cover this procedure A#2190, A#2012 and A#1848.

The TNBS-induced colitis protocol is defined by Loukas Laboratory Protocol Colitis SOP_001 and is adapted from Wirtz *et al.* and Ruyssers *et al.* [5, 6]

Safety/Notes: Full PPE including gown and gloves is recommended. TNBS is an irritant and appropriate safety procedures should be adhered to. Appropriate safety procedures should be adopted when using scalpel blades and syringes and appropriate disposal of sharps is required.

The administration of anaesthetic as described in this procedure requires a current DS4-DS8 approval from the Queensland Government. A copy of this approval is held by the facility.

This procedure also requires induction into the SPF facility, instruction in the correct handling of small mammals, the record of which will be kept by the manager of the facility.

Adherence to SPF facility safety and requirements is mandatory.

Materials/Instrumentation:

Equipment Requirements:

- Laminar Flow Cabinet
- Access to an approved SPF facility

Consumables and reagents:

- Microfuge tubes
- 0.22 µm filter cartridges - Millex syringe filter unit 33 mm/4 mm
- Sterile syringes, 19G, 21G and 23G needles
- Insulin syringes

• TNBS (2,4,6-Trinitrobenzenesulfonic acid solution), also known as picrylsulfonic acid solution (5% w/v H_2O) (Sigma)

• Ethanol

• DPBS

• Viscotears® gel (Novartis)

• KY-Jelly (Johnson & Johnson)

• Insyte Autoguard Shielded IV catheter 20G x 1" Pink (Becton Dickinson) – discard all but the flexible catheter for this procedure.

• Ketamil

• Xylazil

• Trizol – (Invitrogen)

• Complete media (see ELISA procedure).

Method:

Sourcing and husbandry conditions: Animals are supplied by ARC (commercial breeding facility, Perth, WA). The strains of mice used for these experiments will be male C57BL/6 4-6 weeks of age or male BALBC 4-6 weeks of age (strains are not mixed in either experiments or replicates).

Mouse supplied are age and weight matched, assessed for health and placed at random in cages of up to 5 animals per cage. Treatment groups can be mixed within a cage however, mice in the negative control treatment group (naïve mice) are kept separately to prevent cross contamination with TNBS. Mice are rested for 5-7 days prior to the start of the experiment.

Animals are kept in a SPF facility and are provided with mouse chow and water *ad libitum*. They are exposed to a 12-hour day/night cycle and are kept in a temperature/humidity-controlled environment. Bedding is changed weekly and enrichment is provided to all cages. Animals are visually checked during settling in process and monitored daily during the course of the experiment.

Experimental

General Notes – The TNBS colitis model is a qualitative model and experimental conditions can vary from batch to batch of TNBS. The aim of this experiment is to

induce weight loss and symptoms similar to an acute colitis episode. There may be variation from lot to lot of TNBS reagent and each lot should be validated by inducing colitis in a small number of mice. Please refer to the workflow for the important time points of this experiment.

1. **Preparation of TNBS** – TNBS is administered at 2-3 mg/mouse dissolved in 40-60% ethanol – exact ratios determined with each new lot of TNBS. TNBS is stored frozen and is light sensitive and should be prepared immediately prior to administration and wrapped in aluminium foil. TNBS is administered rectally at a rate of 100µL per mouse.
2. **Preparation of material for screening** – All mouse injections need to be prepared aseptically in a laminar flow cabinet. Material that cannot be heat sterilised is to be filtered through a 0.22 µm filter.
3. **Preparation of anaesthetic** – Anaesthetic is prepared aseptically to a concentration of 50 mg/kg of Ketamil and 5 mg/kg of Xylazil is prepared in sterile PBS at a rate of 200µL per mouse. Prepared material is to be stored at 4°C.

Experimental procedure

1. **Administration of screening material** – Inject animal with a single intraperitoneal injection with a 29-gauge insulin needle in the right lower quadrant (total volume = 200µL (concentration 10-50µg per animal of LPS-depleted material) between 5-6 hours before TNBS administration. Animals are gently restrained during injection using a scruff pad and returned to the appropriate cage to rest prior to procedure.
2. **TNBS Administration** - Mice are anesthetized with 50 mg/kg of Ketamil and 5 mg/kg of Xylazil prepared in sterile PBS; 200µl is administered by intraperitoneal injection with a 29-gauge insulin needle in the right lower quadrant. Animals are gently restrained during injection using a scruff pad. Once the mice are unresponsive to stimulation, Viscotears® gel is applied to the eyes. Draw up 100µL TNBS (2-3 mg/mouse) mixed with 40-60 % ethanol with a syringe attached to the catheter for delivery to the mouse, lubricating the rectum and catheter with a liberal amount of K-Y jelly. Invert the mouse and gently insert the catheter into the colon of the mouse and slowly expel the TNBS solution. Invert the mouse for two minutes to prevent anal leakage. Return the mouse to the cage and monitor until they wake up 20-30 minutes later.

3. **Monitoring and Measurement** - Mice will be monitored daily for 3 days post-injection for weight loss and general appearance according to the following scoring criteria:
    - Weight loss, no change or increase = 0; < 5% = 1; 6 – 10% = 2; 11 – 20% = 3; > 20% = 4.
    - Faecal Output, normal = 0; pasty, semi-formed = 1; past, some blood = 2; bloody liquid = 3; unable to defecate after 5 minutes = 4.
    - General appearance (piloerection and mobility), normal = 0; piloerection only = 1; lethargy and piloerection = 2; motionless, sickly = 3. Quality of fur and grooming behaviour should be observed as well as social interaction and curiosity for mobility scores.

This information is recorded on a worksheet (see below for example).

Group	Clinical score								Macroscopic score								
	Day 1				Day 2				Day 3								
	Wt	Mob	Fur	Fecal	Wt	Mob	Fur	Fecal	Wt	Mob	Fur	Fecal	Colon len	Adhesion	Edema	Thicken	Ulceration
Naive																	
1																	
2																	
3																	
4																	
5																	
TNBS																	
1																	
2																	
3																	
4																	
5																	
Sample X																	
1																	
2																	
3																	
4																	
5																	
Sample Y																	
1																	
2																	
3																	
4																	
5																	

Figure 3.2 Example of a clinical/macroscopic score sheet for recording data for TNBS colitis experiment.

In the normal course of TNBS administration, exposed animals weight loss ceases by day three of the experiment. Any animals that have more than 30% weight loss over a 24-hour period or are displaying undue distress are to be sacrificed. Any unexpected losses during this experiment must have an “Adverse Event Form” lodged with the ethics committee through laboratory channels.

4. **Sacrifice and collection of material** - At the end of the experiment all animals will be sacrificed using CO_2 asphyxiation and tissues for assessment collected. After the harvesting of tissues, the remainder of the mouse will be autoclaved and disposed of through JCU approved routes.

   The colon length is measured and assessed for length, oedema, adhesion and ulceration. Pictorial evidence is recommended; if possible publication quality photos should be taken.

   At sacrifice a number of tissues can be collected for monitoring the effects of the treatment, serum, draining lymph nodes, spleen, and gut tissue can be collected.

   Colon

   A portion of the gut tissue (caecum to anus) can be dissected into four pieces:

   1) One piece will be cultured *'ex vivo'* and the immune response assessed by measurement of cytokines released into culture supernatants. The weight of this tissue is recorded in mg.

   2) One piece will be flushed with PBS and then fixed in formaldehyde, blocked in paraffin and sectioned and stained. These sections will then be used to determine infiltration of inflammatory cells to the intestine. Slides are stained with H&E; inflammatory infiltrate is determined using a modified version of the scoring method described by Hong *et al* .[392].

   3) One piece will be preserved in Trizol for RNA isolation and subsequent conversion to cDNA so that real time PCR can be performed using primer pairs for inflammatory cytokine mRNA expression.

   4) The final piece of colon tissue will be snap frozen and later homogenized and analysed for tissue cytokines. The weight of this tissue is recorded in mg.

Experimental Workflow

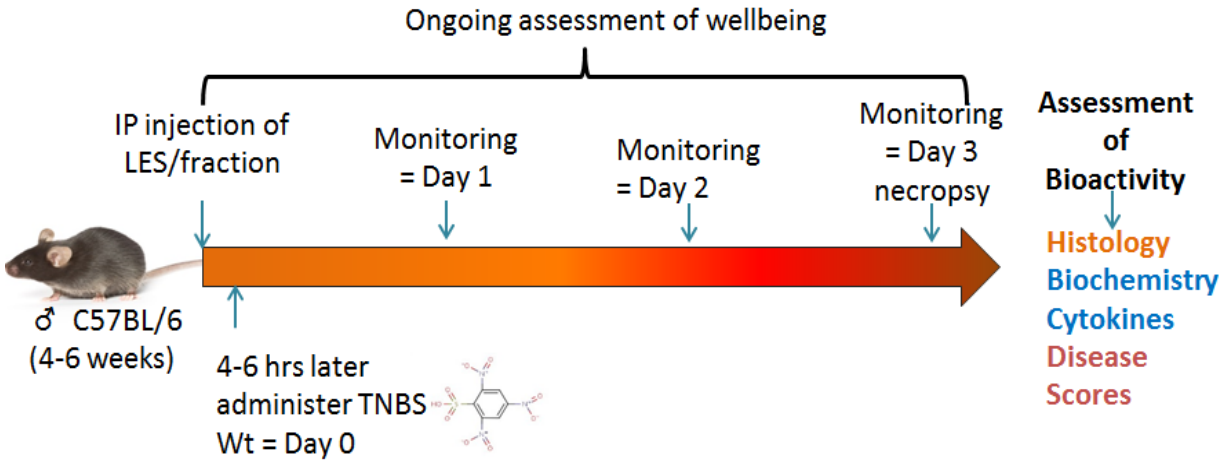


Figure 3.3 Schema of TNBS induced chemical colitis workflow.

3.1.5 Procedure: Histology procedures

Scope: This procedure covers the preparation of sample samples, transport, and interpretation and scoring of histology slides for this project. Histology is carried out by commercial facilities – QIMR Berghofer Histotechnology Services or QML Pathology Laboratory.

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate safety procedures should be adopted when using scalpel blades and appropriate disposal of sharps is required. Appropriate disposal of solvents is required.

Materials/Instrumentation:

Equipment Requirements:

- Automatic pipettes
- Aperio image reader (freeware download) (Aperio\ImageScope\ImageScope.exe)
- Stereomicroscope with x10 and x40 ocular lenses
- Digital camera for recording images

Consumables and reagents:

- 10% buffered Formalin
- Microfuge tubes
- 70% ethanol

Method:

1. Aliquot 1 mL of 10% buffered formalin into a Microfuge tube. Add tissue and fix overnight for 24 hours. Fixing time may be truncated if immunohistochemistry staining is desired.
2. Transfer tissue to 1 mL of 70% ethanol for storage prior to histology processing.
3. Arrange for processing in either of the commercial facilities for staining. Generally staining is limited to haematoxylin and eosin (H&E) or Periodic acid–Schiff (PAS).
4. Tissue is embedded in paraffin and sectioned longitudinally for histology at 4-µm thickness.
5. Slides are de-identified, and scoring is done in a blinded fashion.
6. High-resolution images of slides are viewed using the Aperio image reader (freeware download) (Aperio\ImageScope\ImageScope.exe) or viewed under an x40 magnification. Images of representative areas for each group are recorded.
7. Cross-sections of colons are scored as follows: Ulceration (no ulcers = zero; 1 ulcer = 1; 2 ulcers = 2; 3 ulcers = 3; and >3 ulcers = 4). If ulcers are >200 µm in diameter, scoring is based on 200 µm intervals. Infiltration (0 = no infiltrate, 1 = infiltrate at crypt bases, 2 = infiltrate reaching to muscularis mucosa, 3 = extensive infiltration reaching the muscularis, and 4 = infiltration of the submucosa with oedema). The integrity of the epithelium is scored (0 = normal morphology, 1 = loss of goblet cells in one area, 2 = loss of goblet cells in more than one area, 3 = loss of crypts in one area, and 4 = loss of crypts in more than one area). Lymphoid follicles are also scored (none = 0, 1 = 1, 2 = 2, 3 = 3, >3 =

4). The aggregate score for each treatment group is determined for a maximum score of 16.

8. PAS staining is assessed by determining the number of goblet cells in 200 µm intervals.

3.2 RESEARCH AIM 2: Investigation of Immune cell activation by small molecule fractions

This section covers

- Sample preparation for *ex vivo* colon culture and tissue homogenate,
- Cytokine assays – *ex vivo* culture and tissue homogenate
- Signalling pathway assays – tissue homogenate
- PCR- assays – Trizol preserved tissue

3.2.1 Procedure: Sample preparation for ex vivo colon culture and tissue homogenate

Scope: This procedure describes the general procedure s preparation of *ex vivo* tissue culture and tissue homogenate for biochemical and immunological assessment.

Safety/Notes: Full PPE including gown and gloves is recommended.

Materials/Instrumentation:

Equipment Requirements:

- Microfuge Centrifuge –suitable for Microfuge tubes
- gentleMACS™C Tubes
- gentleMACS tissue DIssociator
- Tissue grinder
- balance
- Access to 4°C cold room

Consumables and reagents:

- Microfuge tubes

- 24 well cell culture plate
- PhosSTOP™ tablets (Sigma-Aldrich) – reconstituted as per instructions
- Sterile PBS
- **Complete Media** (500 mL total volume)- to 400 mL RPMI (Gibco, Life Technologies), 50 mL 10% Foetal Bovine Serum (heat inactivated) (Gibco, Life Technologies), 10 mL 20mM HEPES (Gibco, Life Technologies), 0.5 mL 1X 2-Mercaptoethanol (Gibco, Life Technologies), 7.5 mL 1.5 mM Sodium pyruvate (optional) (Gibco, Life Technologies), 5 mL Gentamicin (50µg/mL) (Gibco, Life Technologies) – make up to 500 mL with RPMI and filter through a 0.22 µM Durapore Stericup (Millipore).

Preparation of samples:

NOTE: All tissues should be weighed prior to processing; this allows for the standardisation of all results to unit/mg of tissue. Repeated freeze thaw cycles should be avoided on samples.

***Ex vivo* tissue cultures** –

1. After collection, each piece of tissue is weighed in mg and transferred to a well of a 12(24?) well tissue culture plate with 500 µL of complete media
2. Tissue is incubated at 37°C for 24 hrs.
3. The supernatant is then transferred to a labelled Microfuge tube and stored at -80°C until required. Samples are thawed on ice prior to assay.
4. Thawed samples should be centrifuged at XXXX *g* prior to use for analysis.

Tissue homogenates –

1) Tissues are snap frozen in liquid nitrogen and stored at -80°C until required for assay.
2) Defrost on ice. Determine the weight of the tissue and record in mg.
3) Prepare PhosSTOP™ as described and homogenize tissue in 200 – 2000 µL of PhosSTOP™ by one of two methods outlined below. Keep tissues and buffers ice cold during this process.
   a) Use the gentleMACS™C tubes and the gentleMACS Dissociator. Follow Insert instructions and use the spleen cycle to disrupt colon tissue. Transfer to Microfuge tube.

b) Using a glass tissue grinder with 200-500µL of PhosSTOP™ buffer, grind tissue on ice completely. Transfer to Microfuge tube.

4) Centrifuge homogenate and transfer supernatant to a fresh tube for assay. Homogenate can be stored on ice or frozen at -80°C

3.2.2 Procedure: ELISA assay method

Scope: This procedure describes the general procedure for the determination of cytokine levels using eBioscience Ready-Set-Go! assay.

Safety/Notes: Full PPE including gown and gloves is recommended.

Materials/Instrumentation:

Equipment Requirements:

- Microfuge Centrifuge –suitable for Microfuge tubes
- Microplate Spectrophotometer – Polarstar Omega – BMG Labtech
- Access to 4°C cold room
- Multi-channel pipette
-

Consumables and reagents:

- ELISA kit (eBioscience Ready-Set-Go) for cytokine containing appropriate antibodies, diluents, and buffers.
- Stop solution – 2N H_2SO_4
- Wash solution – 1x PBS and 0.05% Tween-20 (Sigma –Aldrich)
- Microfuge tubes
- 96 well Nunc plate suitable for assay

Method:

1. The following cytokines are measured using the following general procedure; IL-1β, IL-4, IL-5, IL-6, IL-13, IL-12p70, IL-17, IFN-γ, TNF and TGF-β. Individual

variations in procedures are outlined in the package inserts as supplied by eBioscience.

2. A 96 well plate is coated with the appropriate capture antibody (100 µL) and incubated at 4°C overnight.
3. Wash wells three times with wash buffer then tap plate to remove residual buffer.
4. Add 200µL of 1x Assay diluent to block wells. Incubate at room temperature for 1 hour.
5. Wash wells as per step 3.
6. Add 100 µL of relative standard (see Table 3.1 for concentration) in duplicate, carrying out serial dilutions (x8) to below the detection limit of the relative cytokine; include blank. Load 100 µL of each sample, including duplicates and dilutions as required. Cover plate and incubate at room temperature for two hours (or overnight at 4°C).
7. Wash the wells five times then add 100µL of detection antibody. Cover plate and incubate at room temperature for an hour.
8. Wash the wells five times then add 100 µL of Avidin-HRP to each assay well. Cover plate and incubate at room temperature for 30 minutes (in the dark).
9. Aspirate the wells and wash seven times (each wash should soak for one to two minutes) and tap to remove any excess liquid from the well.
10. To develop the well add 100 µL TMB reagent (supplied in kit) and incubate in the dark for 10-15 minutes. Add 50 µL of stop solution – well should turn from blue to yellow.
11. Determine concentrations by reading in a plate reader at 450 nm (corrected by the 570 nm reading). Determine the cytokine concentration in pg/mL (normalise by tissue weight if available).

Cytokine	Standard concentration
IL-1β	1000 pg/mL
IL-4	500 pg/mL
IL-5	500 pg/mL
IL-6	500 pg/mL
IL-13	500 pg/mL
IL-12p70	4000 pg/mL
IL-17A	500 pg/mL
IFN-γ	2000 pg/mL
TNF α	1000 pg/mL
TGF-β	1000 pg/mL

Table 3.1. Concentrations for the upper standard for each cytokine assayed, as per the technical data sheet from eBiosciences.

3.2.3 Procedure: Tissue p-IκB-α (Ser32) and p-NF-κB p65 (Ser536) assay method

Scope: This procedure describes the general procedure for the determination of the levels of phosphorylated inhibitor of κB-α (Ser32) (p-IκB-α (Ser32)) and phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells p65 (Ser536) (p-NF-κB p65 (Ser536))

Safety/Notes: Full PPE including gown and gloves is recommended.

Materials/Instrumentation:

Equipment Requirements:

- Microfuge Centrifuge –suitable for Microfuge tubes
- Microplate Spectrophotometer – Polarstar Omega – BMG Labtech
- Access to 4°C cold room
- Multi-channel pipette

Consumables and reagents:

• **p-IκB-α (Ser32) and p-NF-κB p65 (Ser536)** Pathscan kits (Cell Signalling Technology) containing appropriate antibodies, diluents, and buffers.

• 96 well Nunc plate suitable for assay

Method:

1. Procedures are outlined in the package inserts as supplied by Cell Signalling Technology.
2. Microwell strips coated with the appropriate capture antibody (100µL) and incubated at 4°C overnight.
3. Load 100µL of each sample, including duplicates and dilutions as required. Cover plate and incubate at 37°C for two hours (or overnight at 4°C).
4. Wash the wells four times then add 100µL of detection antibody. Cover plate and incubate at 37°C for an hour.
5. Wash the wells five times then add 100µL of HRP to each assay well. Cover plate and incubate at 37°C for 30 minutes (in the dark).
6. Aspirate the wells, wash seven times, and tap to remove any excess liquid from the well.
7. To develop the well add 100µL TMB reagent (kit supplied) and incubate at 37°C in the dark for 10-15 minutes. Add 100µL of stop solution – well should turn from blue to yellow.
8. Determine concentrations by reading in a plate reader at 450 nm. Determine the concentration as relative absorbance (normalise by tissue weight if available).

3.2.4 Procedure: Reverse Transcription - Polymerase Chain Reaction (RT-PCR) method (Trizol)

Scope: This procedure covers the preparation of sample and RT-PCR assay.

Safety/Notes: Full PPE including gown and gloves are to be used. Appropriate safety procedures are to be adopted and appropriate disposal adopted when Trizol™ is used. Procedure should be carried out in a fume hood cabinet where possible and all solvents should be disposed of appropriately. Procedure should be carried out on the RNA bench using suitable precautions to avoid contamination.

Materials/Instrumentation:

Equipment Requirements:

- Nanodrop
- Heating block/waterbath
- Tissue Dissociator – Qiagen
- Rotor-Gene

Consumables and reagents:

- RNAse free tips and plasticware.
- Chloroform
- 75% Ethanol
- 100% Isopropanol
- DEPC Water (RNAse free)
- Qiagen kits for SYBR® Green-based RT-PCR kits
- Primers for tRNA of interest plus those for housekeeping (HK) genes (Quantitect® Primer Assay (Qiagen)
- RNAseOUT™
- Superscript™ III RT

* All equipment and consumables should be RNA (do you mean RNase?) and DNA (DNase? free.

Step 1: Sample preparation – RNA isolation: Trizol™ method

Note: Unless otherwise indicated keep samples on ice for this method.

1. Defrost sample (50-100 mg of tissue) on ice and transfer tissue and 1 ml Trizol™ to a flat bottomed 2 mL Microfuge tube. Add a stainless-steel ball to the tube.
2. Using the Qiagen Tissue disruptor on 25 x 1000 rpm for 2 minutes x 2
3. Add 0.2 mL of chloroform to each tube. Shake tubes for 15 s and incubate at room temperature for 2-3 minutes.
4. Centrifuge 4°C/12000 x *g*/15 minutes.
5. Remove the clear top layer containing the RNA and place in a fresh 1.6 mL Microfuge.
6. Add 0.5 mL of 100% isopropanol to this tube and incubate at room temperature for 10 minutes.
7. Centrifuge 4°C/12000 x *g*/10 minutes.
8. The RNA should have precipitated into a pellet. Carefully remove supernatant and wash pellet with 1 mL of 75% Ethanol. Quickly vortex tube and centrifuge 4°C/7500 x *g*/5 min.
9. Dry pellet carefully and resuspend pellet in 50µL of RNAse free water.
10. Incubate tube in a heating block/waterbath 55-60°C/10-15 minutes. Sample can be stored at -80°C at this stage
11. Determine the concentration and purity of the RNA and make up 10 µL of 1 µg/µL for cDNA synthesis.

Step 2: cDNA synthesis

1. Assemble a total volume of 13µL of the following; 1µL of oligo (dT) 20 [50 mM]; 1µL dNTP mix [10 mM]; 10 pg-5µg of total RNA with the remainder RNAse free water.
2. Incubate at 65°C for 5 minutes. Place on ice for 1 minute.
3. Pellet the tube contents briefly in a microfuge then add; 4 µL 5x First strand buffer; 1µL DTT [0.1 mM]; 1µL RNAseOUT™; 1µL Superscript™ III RT [200 U/µL] to make a total volume in the tube of 20 µL. Mix gently by aspiration.

4. Incubate 50°C for 30-60 minutes.
5. Inactivate the reaction by incubating 70°C for 15 minutes.
6. Determine the concentration and purity using nanodrop. Determine the purity of the cDNA and volume required concentration for 100 ng of cDNA for RT-PCR.

Step 3: RT-PCR using Rotorgene

Quality control

Make up super pool of all RT-PCR samples (1µL of each). Make the following dilutions 1:10, 1:100, and 1:1000 to determine the cycle time (Ct) values. Quality control is done on one gene usually a housekeeping gene. Ct values that fall between 18-24 are desirable and indicate optimal dilution.

Sample analysis

1. Sample concentration should not exceed 100 ng of starting material in a total reaction volume of 10µL.
2. In a 100µL Rotor-gene tube add 2 x Rotor-gene SYBR Green PCR Master Mix, 5µL; 10x QuantiTect Primer Assay, 1µL (0.5µL Forward primer; 0.5µL Reverse primer); sample cDNA template (variable volume) (e.g. 1µL of 100 ng cDNA); RNAse free water to make up to the final volume of 10µL.
3. Primers available for the following Assays; Mm_IL10_1_SG(IL10); Mm_Tnf_1_SG(TNF); Mm_Actb_1_SG(Actb)(HK gene); Mm_IL2_1_SG(IL2); Mm_Ifng_1_SG(IFN-γ); Mm_IL17a_1_SG(IL17a); Mm_IL1b_2_SG(IL1β); Mm_IL6_1_SG(IL6); Mm_IL13_2_SG(IL13).
4. Load and run the Rotorgene on a 3-step melt using the following settings (84 minutes run). Hold 95°C/10 minutes; Cycling (40 cycles) of 1) 95°C for 10 sec, 2) 60°C for 15 sec, 3) 72°C for 20 sec (cycling A); Melt ramp 72°C →95°C at 1°C per step, hold 5 seconds.
5. Record the M value from the RT cycler

3.3 RESEARCH AIM 3: Separate LES into fractions and determine which of these confer protection in TNBS colitis.

This section covers

- Polarity extractions for sample separation.
- TLC separation for characterisation.

3.3.1 Procedure: Polarity extraction method

Scope: This procedure covers the preparation of sample for GCMS or bioactivity screening via the TNBS colitis method. This procedure can be applied to lyophilised samples or liquids. Extractions should be carried out in order of increasing polarity.

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate safety procedures should be adopted when using scalpel blades and appropriate disposal of sharps is required. Procedure should be carried out in a fume hood cabinet and all solvents should be disposed of appropriately.

Materials/Instrumentation:

Equipment Requirements:

• Separation flasks*
• Schott bottles*
• Glass beakers*
• Rotary evaporator

* All glassware is to be pyrogen-free and appropriately cleaned (rinsed with acetone).

Consumables and reagents:

• Ashless filter paper (Whatman)
• Chloroform (Chem Supply – HPLC grade)
• Ethyl Acetate (Chem Supply – HPLC grade)
• Petroleum Spirits 40-60°C (Chem Supply – Analytical reagent)
• Methanol (Sigma-Aldrich – HPLC grade)
• Acetone (Vetec – Analytical Reagent)

- Dichloromethane (DCM)
- Dimethyl ethylene (DME)

Note: Avoid plastic when carrying out polarity extractions to limit the leaching of phthalates and other compounds from plastic into the extraction.

Method A – liquid sample:

General method

1. Defrost sample for polarity extraction (approximately ~50 mL) and store at 4°C until ready for extraction.
2. Make up a 2:1 solution of chloroform: methanol – chloroform 4 mL: methanol 2 mL (CM). Otherwise aliquot 6 mL of the desired solvent.
3. Using a separating flask combine 50 mL of solution of interest and 6 mL of the extraction fluid and mix by inversion 30-50 times (vent periodically) until thoroughly mixed. Allow phases to separate and remove the bottom layer, ensuring that there is no aqueous layer in the decanted material.
4. Repeat step 3 two times making a total of approximately 18 mL of extracted solutions.
5. Solutions are reduced in volume on the rotary evaporator.
6. Small volumes of ~500µL are dried down under nitrogen (small volume method B).

Method B – dried sample:

General method

1. Lyophilised material (~300 mL of original volume lyophilised to dryness).
2. Make up a 2:1 solution of chloroform: methanol – make approximately 15 mL of chloroform: methanol solution. Otherwise aliquot 15 mL of the desired solvent.
3. Extract lyophilised material with 13 mL of the chloroform: methanol (or solvent) solution. Use a glass stick and ensure that solid material is mixed with the extraction solution. Rest the solution for approximately 15 minutes. Filter through Whatman filter paper to remove solids. Rinse with 2 mL of the chloroform:

methanol extraction material. Rinse beaker with a small amount of DCM (or matched solvent)

4. Transfer material to sample vial and dry under nitrogen to concentrate.
5. Repeat step 3 two times making a total of approximately 18 mL of extracted solutions.
6. Solutions are reduced in volume on the rotary evaporator.
7. Small volumes of ~500 µL are dried down under nitrogen (small volume method B).

3.3.2 Procedure: Thin Layer Chromatography and band extraction method

Scope: This procedure covers the optimisation of thin layer chromatography (TLC) and extraction of bands for further testing. To simplify this method samples should be polarity extracted prior to running on TLC to group in to similar polarity.

Safety/Notes: Full PPE including gown, gloves is recommended. Appropriate safety procedures should be adopted using when using scalpel blades and appropriate disposal of sharps is required. Procedure should be carried out in a fume hood cabinet and all solvents should be disposed of appropriately.

Materials/Instrumentation:

Equipment Requirements:

- Separation flasks*
- TLC tank (with lid)
- Schott bottles*
- Glass beakers*
- UV light for visualisation (254nm, 365 nm – Spectroline Model CM-10A)

* All glassware is to be pyroneg free and appropriately cleaned (rinsed with acetone and optimised solvent system).

Consumables and reagents:

- Ashless filter paper (Whatman)
- Silica Plate – Analtech-Unitplate Chromatography TLC plate (Cole-Palmer 34103-05) Silica Gel GF preparative layer with UV254 (Lot # 30014 (Cat # 02013)
- Chloroform (Chem Supply – HPLC grade)
- Ethyl Acetate (Chem Supply – HPLC grade)
- Petroleum Spirits 40-60 °C (Chem Supply – Analytical reagent)
- Acetonitrile (ACN)
- Ammonia (30%)
- Methanol (Sigma-Aldrich – HPLC grade)
- Acetone (Vetec – Analytical Reagent)
- Dichloromethane (DCM)
- Dimethyl ethylene (DME)
- Petroleum Spirit 40-60 °C

TLC Optimisation:

Rationale:

The separation system for TLC needs to be optimised to ensure the maximal separation of components of the sample. A polarity extraction prior to TLC is recommended to ensure maximal separation of components in the mobile phase.

Optimising solvent system for TLC

1. In small beakers set up approximately 5 mL of mobile phase. Using the polarity extraction as a guide make up a variety of solvent combinations of spot a small amount of the sample of interest on origin of small portions of silica gel plate (2 cm x 5cm) at the origin.
2. Initial separation is trialled in a single solvent system. Optimisation is judged by maximal separation of components as judged by visualisation at 254 and 365 nm.
3. A mixed solvent system is subsequently trialled 50:50 combinations of chloroform:methanol, ethylacetate:methanol, chloroform:ethylacetate again assessing separation by visualisation at 254 and 365 nm.

4. A final optimisation step is to trial different proportions of solvent mixture. In the case of this study a chloroform:methanol solvent system proved the most effective and the following ratios are trialled:

Chloroform %	Methanol %
95	5
90	10
80	80
70	30

5. The last step in optimisation is to select two solvent systems and trial them along each other and then select the optimal separation for all compounds as visualised at 254 and 365 nm.

Large plate TLC Separation

Note: It is important to proceed to extraction from the silica plate quickly after this separation to avoid sample binding and loss. Have equipment and supplies prepared prior to commencing this separation.

1. Prepare a large TLC tank by cleaning thoroughly with acetone. All solvent systems are to be made up fresh and loaded just before running separation. The final step of solvent system preparation is addition of ammonia. This should happen in the tank and gently mixed. A wick of filter paper is added to the tank is then covered and the system is left to equilibrate prior to loading plate.

2. The solvent system used in this extraction is choloroform:methanol (70:30) ratio (70 mL with 60 drops of ammonia)

3. The sample is loaded onto the bottom of the plate (the origin – Rf0) (approximately 2 cm from the bottom). This is done sequentially allowing for drying between applications with up to 50 µg being loaded on the plate. Ensure that the plate is completely dry before loading into the separation system.

4. Carefully place the silica plate into the tank ensuring that it is not in contact with the side of the tank. Replace the lid and run until the solvent front is 7/8- 9/10 up the plate.

5. Remove the plate and let dry briefly. Visualise the plate and outline bands carefully using a soft leaded pencil. Proceed promptly to band extraction.

Band extraction from TLC Separation

1. Carefully cut out each band and subsequently into small pieces and place in separate clean glass beakers each containing dicholormethane:methanol (70:30). Bands closer to the origin are extracted in dicholormethane:methanol (60:40).
2. Let sit for 30 minutes in solvent extraction system then filter carefully to remove silica particles. Transfer to vials and dry under nitrogen and determine the weight of the extract.
3. This material can be prepared for bioactivity screening and characterisation as described in this chapter.

3.4 RESEARCH AIM 4: Characterise LES

This section covers

- Sample preparation methods- zip tip for MS, preparation for HPLC, preparation for LCMS
- HPLC – Agilent 1600 system
- GCMS – Underivatised samples on the Shimadzu
- GCMS – Derivatised samples on the Agilent
- NMR Sample preparation and analysis
- Statistical analysis of data.
- Bioinformatics workflows

3.4.1.1 Procedure: method for de-salting samples using the C_{18} Zip tip™ procedure

Scope: This procedure covers the procedure for de-salting small amounts of sample in preparation for characterisation on the mass spectrometer. This procedure is adapted from the product insert.

Safety/Notes: Full PPE including gown, gloves is recommended. Appropriate disposal of waste is required.

Materials/Instrumentation:

Equipment Requirements:

Automatic pipettes

Consumables and reagents:

• C_{18} Zip tips™

• Wetting solution: 100% ACN

• Equilibration and Wash solution: 0.1 % Trifluoroacetic acid (TFA)

• Elution solution: 1:1 solution of 0.1% TFA: 50% ACN or 1:1 of 1% Formic Acid (FA): 50% ACN

• Microfuge tubes

NOTE: All reagents are HPLC grade.

Method:

1. Using a regular pipette transfer 5µL of elution buffer to labelled Microfuge.
2. Using a C18 Zip tip pipette 10µL of wetting solution and discard into a tissue. Repeat this step.
3. Repeat this with the equilibration solution two times, discarding into a tissue.
4. Pipette up 10µL of sample and pipette up and down repeatedly but gently to adsorb sample into tip. Repeat this with the rest of the sample until the entire sample is processed.
5. Pipette 10µL of wash solution X3 and discard into tissue.
6. Change the pipette to 4µL. Pipette elution buffer (from tube in Step 1.). pipette gently up and down gently several times.
7. Dry down sample in the Speedivac (using small sample lyophilisation method A). Store at -20 °C or prepare for application onto plate for mass spectrometry.

3.4.1.2 Procedure: method for preparing samples for HPLC and LCMS

Scope: This procedure covers the procedure for preparation of samples for HPLC and LCMS analysis.

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate disposal of waste is required.

Materials/Instrumentation:

Equipment Requirements:

Automatic pipettes

Consumables and reagents:

- 0.1 % TFA
- 1% FA
- 0.22μm filter
- syringes
- Glass vials and inserts
- HPLC filtered de-ionised water

NOTE: All reagents are HPLC grade.

Method:

1. All samples to be loaded onto either the LCMS or HPLC platforms must be filtered prior. Using syringe aspirate the sample and then using a 0.22μm filter remove particulate material from the sample.
2. Samples can be prepared in either TFA of FA if they are being prepared from lyophilised material.
3. Consideration should be given to the type of sample being run. An unknown sample may have a high proportion of “sticky” components, so a guard column must be used and care should be taken not to overload the column.
4. Load sample into a glass vial (use an insert if volume is <100μL). A larger volume may be loaded directly via the sample tubing.

3.4.2 Procedure: HPLC method for the detection and separation of components from a mixture

Scope: This procedure covers the preparation of samples and buffers used for the HPLC instrument – Agilent Technologies 1260 Infinity. Samples are to be run as described by the instrument manual.

Safety/Notes: Full PPE including gown, gloves is recommended. Safety glasses must be worn when operating the HPLC instrument. Appropriate safety procedures should be adopted using when using scalpel blades and appropriate disposal of sharps is required.

Materials/Instrumentation:

Equipment Requirements:

HPLC Agilent Technologies 1260 Infinity system

Consumables and reagents:

- C18 Semi-prep and analytical columns (as outlined in table 3.2.)
- Buffer A – 0.05 % TFA
- Buffer B – 30% ACN
- HPLC vials and inserts
- Tubes for fraction collection
- De-ionised water 0.22 μm filtered

NOTE: All reagents are HPLC grade and all solutions used on the HPLC must be 0.22 μm filtered.

Method:

12. Sample preparation – samples are loaded into glass vials or inserts and loaded into position on the automatic sampler of the instrument. If samples contain salt buffers, a de-salting profile is to be set up. Examples of the run methods are set outlined in Table 3.3.
13. **Buffer A** – 0.05 % TFA and filtered through a Durapore Stericup 0.22 μm membrane (Millipore). Buffer can be stored at room temperature

14. **Buffer B** – 30% ACN – 300 mL of ACN is added to 700 mL of dH2O and filtered through a Durapore Stericup 0.22 μm membrane (Millipore). Buffer can be stored at room temperature.

Analytical column
Jupiter 4 μ Proteo 90 Å 250 x 10.00 mm (4 micron), PN-00G-4396-NO, S/N: 648429-1, maximum flow rate 4mL/min
Semi-prep column
Jupiter 4 μ Proteo 90 Å 150 x 200 mm (4 micron), PN-00F-4396-BO, S/N: 639622-3, maximum flow rate 0.4mL/min

Table 3.2. List of columns used for separation and characterisation on the HPLC Agilent Technologies 1260 Infinity series instrument.

15. Gradient profiles were run at a 1-2 % gradient with a 5-minute wash at the beginning of the run to remove buffers and salts from the column. The appropriate λ max was determined for each sample by assessing the absorbance of the sample across the spectrum using the Polarstar Omega. Generally, monitoring absorbance was 214 nm (for peptide bonds), 280 nm (for phenol groups) or an experimentally determined wavelength such as 230 nm (λmax =phenol red)

Analytical column

Sample volume:	20-40μL			
Collection of peaks:	1-minute slices or selective peak collection			
Gradient profile:				
Time (min)	**Buffer A [%]**	**Buffer B [%]**	**Flow (mL)/min**	**Max Pressure**
0	95	5	1.0	400
5	85	15	1.0	400
50	40	60	1.0	400
55	10	90	1.0	400
60	10	90	1.0	400
62	95	5	1.0	400

Semi-prep column

Sample volume:	40-100µL			
Collection of peaks:	1-minute slices or selective peak collection			
Gradient profile:				
Time (min)	**Buffer A [%]**	**Buffer B [%]**	**Flow (mL)/min**	**Max Pressure**
0	95	5		400
5	85	15		400
50	40	60		400
55	10	90		400
60	10	90		400
62	95	5		400

Table 3.3. HPLC methods used for the separation of LES material.

3.4.3 Procedure: The operation of GCMS Shimadzu QP2010 Ultra

Scope: This procedure describes the operation of the gas chromatography mass spectrometer (GCMS) and Flame Ionization Detection (FID) Shimadzu QP2010 Ultra for the separation and identification of molecules.

Safety/Notes: Full PPE including gown and gloves is recommended. Appropriate safety procedures should be adopted using when using solvents and dispose as required.

Materials/Instrumentation:

Equipment Requirements:

GCMS Shimadzu QP2010 Ultra – System No – 4-00081 with AOC-20i Auto-injector

Consumables and reagents:

• Column: Phenomenex Zebron ZB-50 – 30 m x 0.25 mm, Liquid Phase: 50% Phenyl-50% Dimethylpolysiloxane. Temperature Limits: 40-320/340°C isothermal. Part #: 7HG-G004-11

• Glass sample vials

• Solvent carrier system DCM, Chloroform, Methanol, Ethyl Acetate, Petroleum Spirit (all reagents are to be GCMS quality)

• Wash solution Methanol

Method:

Method Run

Load the appropriate method and download parameters. Set up runs in single or batch modes. Load samples onto the auto sampler. At the end of each run put a vial in position -1 and select the Standby method.

Column ZB-50 Oven Temperature Program		
Rate	Temperature (°C)	Hold Time (min)
-	40.0	1.00
45.00	190.0	20.00
12.00	210.0	10.00
7.00	320.0	4.00
7.00	320.0	5.00
Standby		

Table 3.4 Run conditions for samples on the Shimadzu QP2010 Ultra for both MS and FID runs.

Determining Kovat's Indices

This method utilises the method for determining Kovat's indices as described by Girard [393].

3.4.4 Procedure: GCMS – derivatized Polar metabolites

GCMS Derivatization performed on Agilent with a Gerstel autosampler

This analysis is carried out at the Bio21 institute (Metabolomics Australia), University of Melbourne, using methods and workflow standard operating procedures approved by the facility, briefly described as follows:

Materials/Instrumentation:

Equipment Requirements:

Automatic pipettes

Refrigerated centrifuge for microfuges

Consumables and reagents:

- 100% Methanol
- 100% Chloroform
- Extraction solution: (4.5 mL + 1.44 mL Milli-q water+ 30μL 1mM 13C-sorbitol + 30μL 10mM 13C,15N-valine)- enough for 10 samples
- Methoxamine (30mg/mL) in pyridine (Sigma)
- N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA + 1% TCMS) (Pierce Technologies)
- Microfuge tubes
- 200μL pulled point inserts
- 2mL GC/LC sample vials

NOTE: All reagents are HPLC grade

Method - Sample preparation

1. An equal volume of fluid for extraction and chloroform (50μL) are placed in a microfuge tube and vortexed for 10 seconds.
2. Add 150μL of chilled methanol containing internal standards (130μL methanol + 10μL of 1mM 13C-sorbitol + 10μL of 10mM 13C, 15N-Valine per sample.
3. Vortex again and centrifuge samples at 16,100 *g* at 0°C for 5 mins.
4. Place the supernatant into a new 1.5mL tube containing 100μL of MilliQ filtered water, discard any cell debris.
5. Vortex for 30 seconds then centrifuge samples at 16,100 *g* at 0°C for 5 minutes.
6. Finally transfer aqueous phase (top) into a fresh tube.

GCMS derivatization and analysis

1. A 20µL aliquot of sample is loaded into a GCMS microvial insert then evaporated to dryness.
2. The sample is washed with 50µL of methanol and then dried again to ensure sample is completely dry.
3. Tubes are then crimped to seal and loaded onto the GCMS for analysis.
4. Derivatization is an automated process and is carried out by the Gerstel MPS2 XL autosampler robot (Mülheim) with each sample being treated with methoxyamine, in pyridine, (20µL of 30 mg/mL, w/v) and the methoximated metabolites derivatized with BSTFA + 1% TMCS (20µL, Pierce Technologies). The Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer are used to analyse derivatized samples.
5. A 30 m J & W Scientific VF-5ms column (plus 10 m Eziguard pre-column Agilent Technologies) (250 µm internal diameter and 0.25 µm film thickness) is used. An inlet temperature of 250°C and GCMS interface temperature of 280°C and ion source temperature of 250°C is maintained. Helium is supplied at a rate of 1 mL. minute^{-1}. The initial oven temperature is started at 35°C (hold for 2 minutes) and ramped by 25°C.minute^{-1} to 325°C (hold for 5 minutes). Mass spectra are recorded in t 9.19 scans/s over an m/z range of 50–600.

Data processing

1. Data is processed through Bio21 Institute in-house workflows using Metabolomics Australia GCMS alignment and integration software, PyMS python toolkit [394].
2. The peaks identified are then quantitated by automated integration of the area under the peaks using Agilent MassHunter Quantitative analysis software. The peaks are manually visualized, validated, and gap-filled.
3. Any peaks that are not been correctly identified by the software are manually integrated. Following this, the data is exported as an integrated area matrix for statistical analysis. These data sets are supplied in both raw and processed form for this project.

3.4.5 Nuclear Magnetic Resonance (NMR) – Sample preparation and analysis

Materials/Instrumentation:

600 MHz AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany)

Equipment Requirements:

Automatic pipettes

Consumables and reagents:

- NMR tubes with caps 5 mm diameter x 160 mm
- A suitable heavy solvent:
- D_2O
- Methanol-D (Aldrich Lot number: TA2073V)
- Acetonitrile-D3 (ID NMR DLM-53-IOXO Lot number: 12J-112)
- Chloroform-D (Cambridge Isotope Laboratories Inc. DLM-7TA 50S Lot number: 14H-248)

NOTE: All reagents are HPLC grade

Method - Sample preparation

1. Using up to 1 mg of sample dissolve in 450µL of matched solvent. Where possible the sample is dissolved in 450µl H_2O and 50µl D_2O,
2. In a NMR tube add 50µL heavy solvent and mix thoroughly.
3. Load sample into NMR spectrometer.
4. Analyse sample as guided by technician (analysis profiles have to be established individually but may be repeated after this stage).
5. Perform 2D $^{1}H,^{1}H$ TOCSY, $^{1}H,^{1}H$ NOESY, $^{1}H,^{1}H$ DQF-COSY, $^{1}H,^{15}N$ HSQC, and $^{13}C,^{1}H$ HSQC and acquire spectra at 290 K
6. Samples can be recovered from tubes post analysis.

Note: Step 3 onwards must be performed under the supervision and direction of a fully trained technician.

Sample Analysis

1. To analyse the structures of small molecule components, a 600 MHz AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a cryogenically cooled probe is used and the following spectra were D_2O. 2D $^{1}H,^{1}H$ TOCSY, $^{1}H,^{1}H$ NOESY, $^{1}H,^{1}H$ DQF-COSY, $^{1}H,^{15}N$ HSQC, and $^{13}C,^{1}H$ HSQC spectra are acquired at 290 K.
2. Spectra are recorded with an interscan delay of 1 s. NOESY spectra are acquired using mixing times of 200–300ms, and TOCSY spectra is acquired with isotropic mixing periods of 80ms. Standard Bruker pulse sequences are used with an excitation sculpting scheme for solvent suppression.

3.4.6 Statistical analyses

All statistical analyses are performed using GraphPad Prism (version 6.0 or version 7.0). Where comparisons are made between groups it is the sample treatment + TNBS groups and the TNBS only group; P values of < 0.05 are considered significant *P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001.

When determining the differences, two groups are compared using a Mann-Whitney (unpaired, non-parametric) U test. One-way ANOVA with a Bonferroni test applied (95% confidence interval) is carried out when comparing three or more groups. Results were compared to the TNBS negative control. All results reported denote mean ± standard error of the mean (SEM). All data are representative of at least two experiments. The total n is noted in the respective figure legends and Results section.

3.4.7 Bioinformatics workflows

LC-MS, MS/MS data

Data obtained from analysis platforms is processed through Protein Pilot™ and Mascot™ for sequencing data. Putative sequences are then processed through NIST protein blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and Swissprot (accessed via http://uniprot.org/) both with and without specific species filter to identify peptide matches.

GCMS Data

Underivatized GCMS data are processed using Shimadzu 2010 software. All chromatograms are processed both automatically and manually. Each peak is validated manually and fragment patterns are compared to the NIST spectral library for matches. Fragment patterns for each chemical are compared to the database and consideration is given to both the relative abundance and the probable identity. Matched peaks from separate runs with the same probable identity are assigned an identity. Probable identities of less than 69% are classified as unconfirmed and unknown molecules are identified.

NMR Data

Spectra are compared to published data and chemical identity assigned if matched.

Chapter 4

Media selection

Experimental workflow and rationale

Preamble

In this chapter I present;

The preliminary screening and initial proteomic work up for RPMI-cultured low molecular weight AcES from dog hookworm.

The experimental evidence demonstrates a need for a new method for the culture of dog hookworm *in vitro* for small molecule collection as well as the results of media selection experiments.

Finally, I present the experimental workflow that was the basis of the following chapters on the isolation and characterisation of anti-inflammatory small molecules from AcES.

Outputs from this Chapter:

A collection and culture procedure for *A. caninum* – See Appendix A

4.1 Preliminary screening and proteomic work

This project commenced with access to a large amount of LES reserved from proteomic studies of *A. caninum* cultured in RPMI as previously described [161]. This material was collected from animals in Thailand, Brisbane and locally in Cairns. There were concerns that storage as well the diverse geographic location may result in batches with different molecular composition, and therefore we should be wary of pooling samples. To eliminate this issue each separate collection was run on an analytical HPLC (C18-1% gradient) and overlayed prior to pooling. Typical results can be seen in Figure 4.1. Results show minor differences and it was decided because of these results that samples would be pooled for subsequent work.

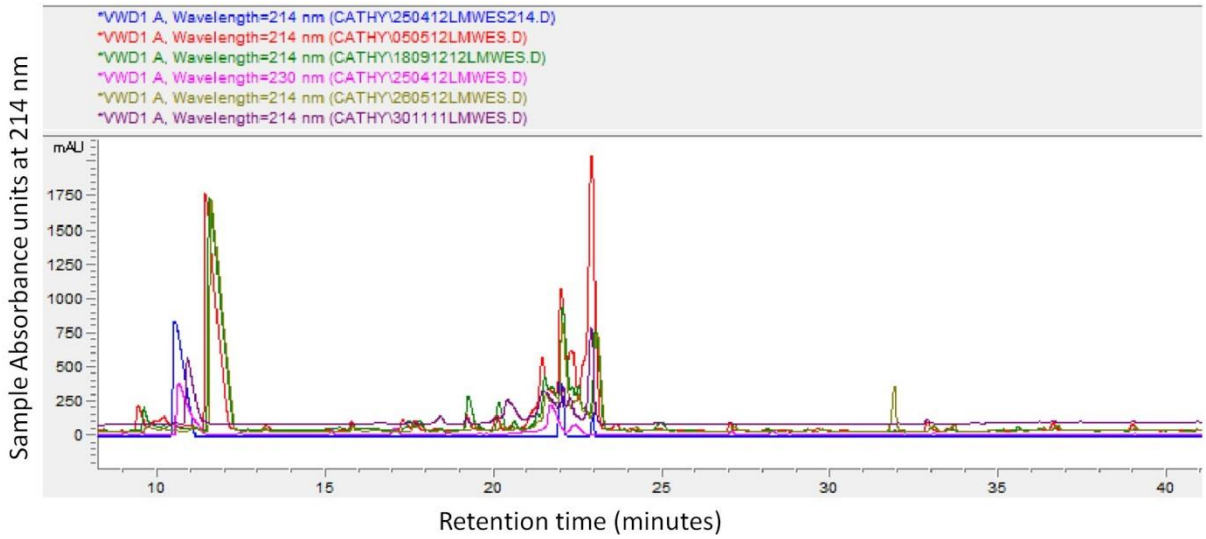


Figure 4.1. An Overlay of absorbance (214 nm) traces of six batches of <10 kDa AcES. The traces show minor component differences. Samples were pooled for screening and characterisation. This is a representative sample only of the 14 batches of AcES compared in this way.

4.2 Peptide component

The initial focus of research effort was on the potential peptides present in the <10 kDa ES from *A. caninum*. The bulk of this research was done on ES obtained from RPMI culture. Samples were prepared and sent to QMIR Berghofer Medical Research Institute (QIMRB) for LC-MS analysis using an ABI 5600 mass spectrometer to identify peptides. These samples included pooled low molecular weight AcES as well as HPLC fractions that had been shown to infer partial protection against inflammation in murine TNBS colitis [373]. In concert with

this, LES was separated by HPLC (Agilent 1600) and peaks were collected for MALDI TOF analysis. Data returned was analysed through Mascot software and putative peptide sequences were aligned to public databases using blast in the NCBIprot and Swissprot databases. Sequences were also aligned specifically to the *A. caninum* draft genome [200].

Putative LES peptide sequences can be found in Appendix B. Most of the peptide sequences identified corresponded to fragments of larger *A. caninum* proteins (100% coverage but low maximum scores) and likely represent proteolytic degradation products of AcES proteins.

I also performed HPLC purification followed by MALDI-TOF MS/MS of LES fractionation peaks that had previously been shown to protect against TNBS colitis [373]. One peptide was identified in all peaks (n=10) and yielded the putative sequence GGPEGPPFLK (See Figure 4.2.). This peptide shared 100% identity (max score 37.5) with the *A. caninum* immunodominant hypodermal antigen Ac16 (Accession ABD98404.1), again corresponding to a small fragment of a larger protein. To investigate the contents of this fraction it was subjected to 1D ^{1}H NMR, however a weak signal meant that the presence of this peptide could not be confirmed.

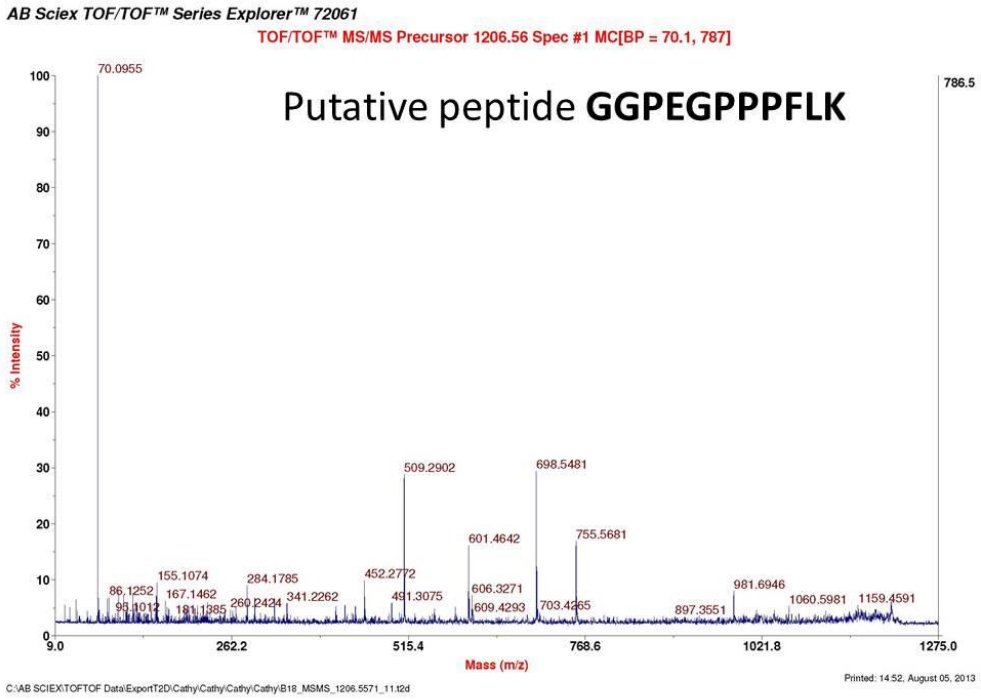


Figure 4.2. The MS/MS peak series of Mw 1206.56 for the putative peptide GGEGPPPFLK.

It likely that the peptides detected here are the degradation products of larger proteins present in AcES. Due to the collection method employed, small peptides produced by the hookworm are likely destroyed by proteases and natural degradation during the culture period (24 hours). As we had observed protection against TNBS colitis from these fractions and we could not definitively find a likely peptide candidate as a final check we carried out 1D ^{1}H NMR on LMW AcES and compared it not only to the RPMI culture fluid alone but to the NMR spectra previously recorded [373]. Figure 4.3. compares these three spectra. It is quite apparent from direct comparison that the peaks in the amide region (7.5-9.5 ppm) are present in the RPMI media alone and corresponds with the signature peaks of phenol red (RPMI pH indicator phenol sulfophalein) which has two peaks at δ 6.62-7.37 [395]. The lack of strong indicators in either Figure 4.3a. or 4.3c. NMR spectra do not support a strong peptide component present in LES.

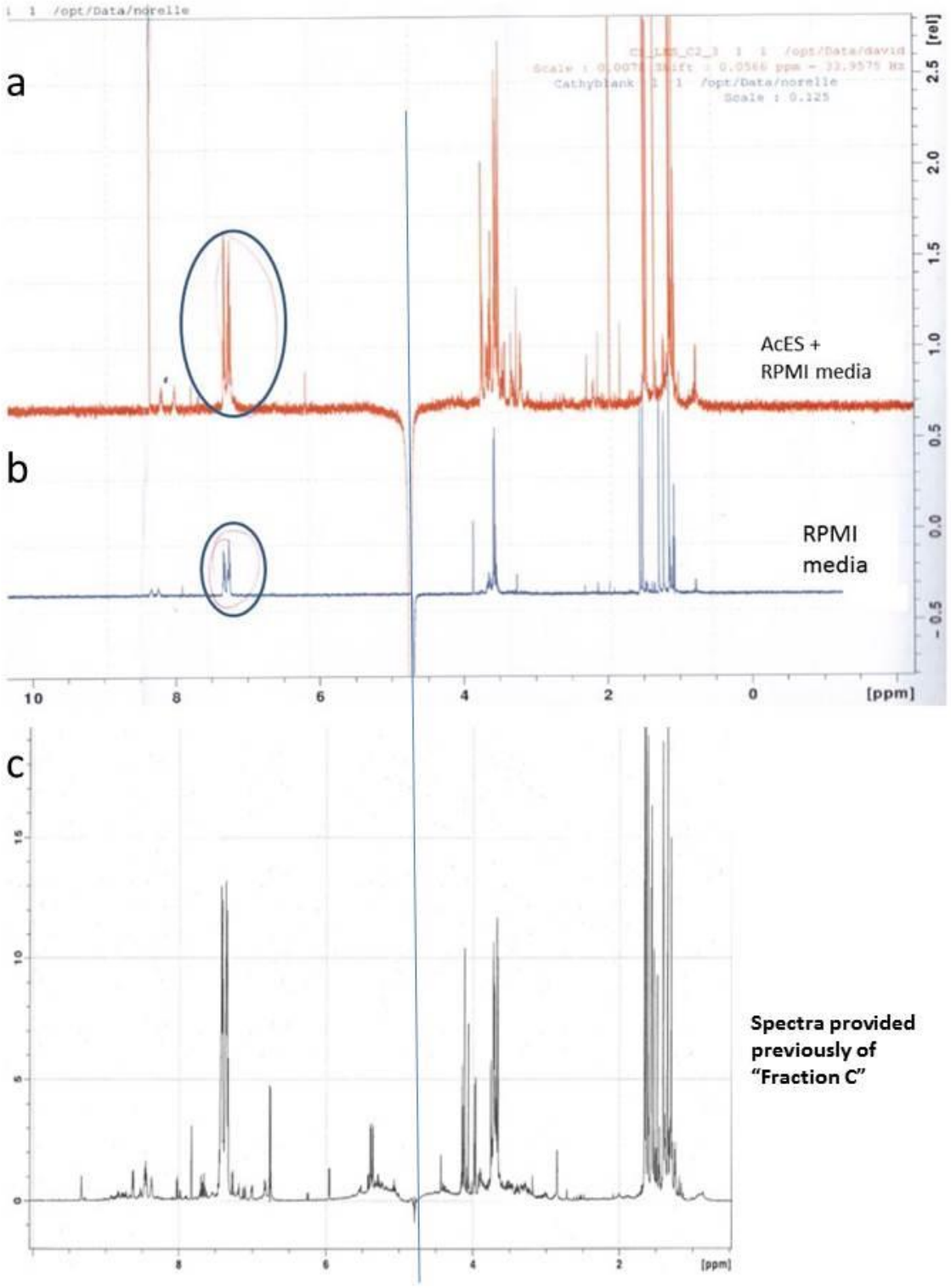


Figure 4.3. **Comparison of 1D ^{1}H NMR spectra** of **a.** LES collected in RPMI media; **b.** RPMI media alone and **c.** spectra published in the thesis of Markus Baumann, 2012 [373] of peak C, the protective fraction identified in his study. The blue circles highlight the peak corresponding to the pH indicator in RPMI media, phenol red.

4.3 Multiple components of RPMI complicate small molecule analysis

The strong spectral absorbance of phenol red overwhelmed other components in LES, as highlighted in Figure 4.4. It was difficult to determine a suitable λ_{max} for media components due to the abundance of phenol red and its high absorbance across the spectra. In addition to this phenol red co-elutes in HPLC with peaks of interest.

In addition to the technical problems associated with phenol red, RPMI contains over 40 ingredients, all of which are detectable using the techniques that I employed. Many of these compounds had reported anti-inflammatory activity. To eliminate the possibility of RPMI components contributing to anti-inflammatory activity in the TNBS colitis screen employed in this study, the therapeutic properties of RPMI culture media was assessed in the mouse model of TNBS colitis. RPMI alone induced significant protection against weight loss induced by TNBS colitis (Figure 5). LES cultured in RPMI afforded no protection in this colitis model, findings that are concordant with previously described results [373].

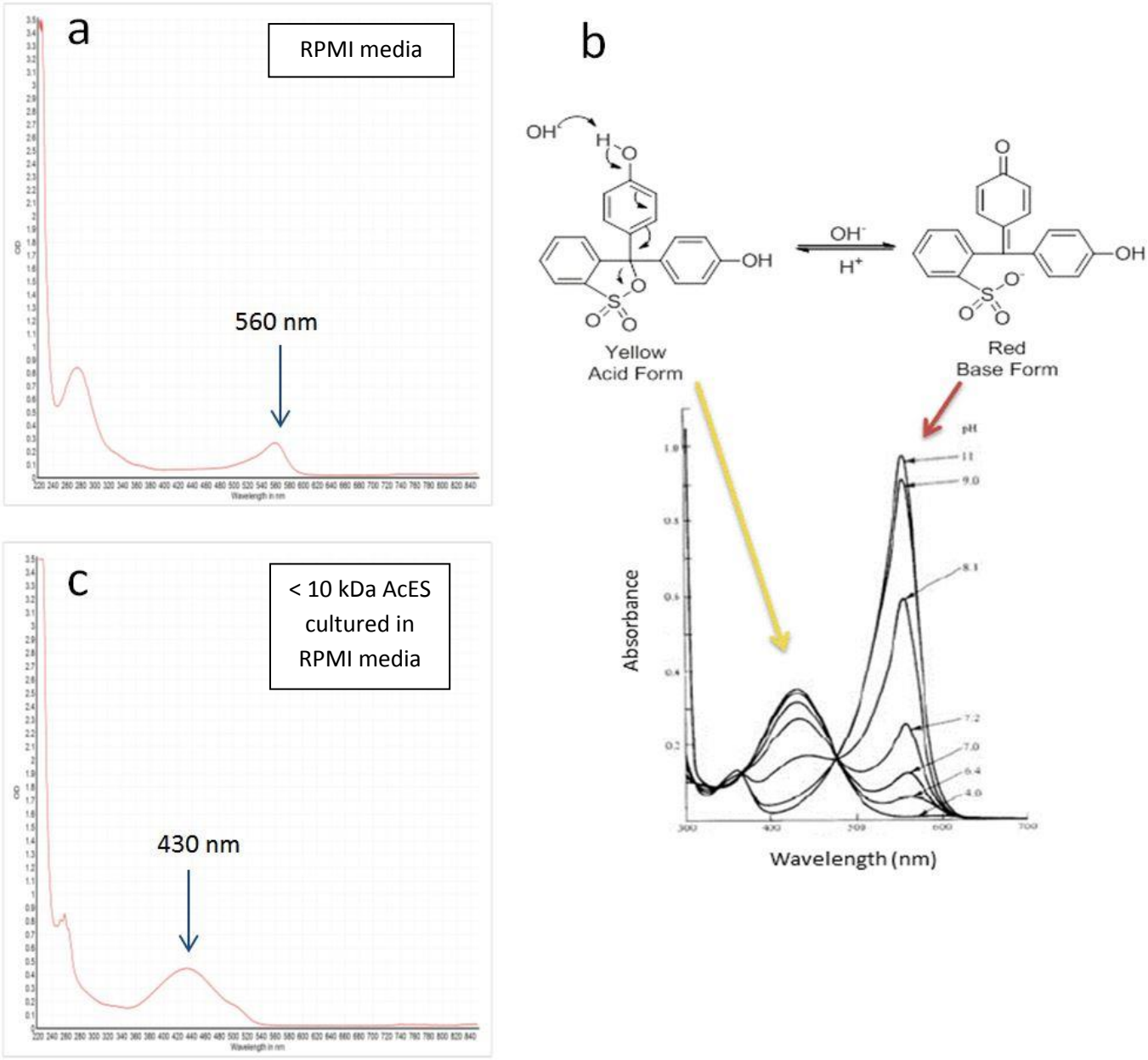


Figure 4.4. Phenol red has an overwhelming spectral presence in RPMI dominating the visual spectrum and contributing to absorbance at the <280 nm. The λ_{max} indicated (red trace) in **a.** and **c.** by the arrows at 560 nm and 430 nm respectively represent those of phenol red at different pH values. **a.** represents the absorbance for RPMI culture material across the visual spectrum **c.** is the visual spectrum (nm) absorbance for low molecular weight AcES cultured in RPMI. These spectra show both a pH change and the contribution of phenol red indicator to the overall absorbance. **b.** represents spectral changes of phenol red across the spectrum as the pH changes. The λ_{max} for phenol red changes as the media becomes more acidic

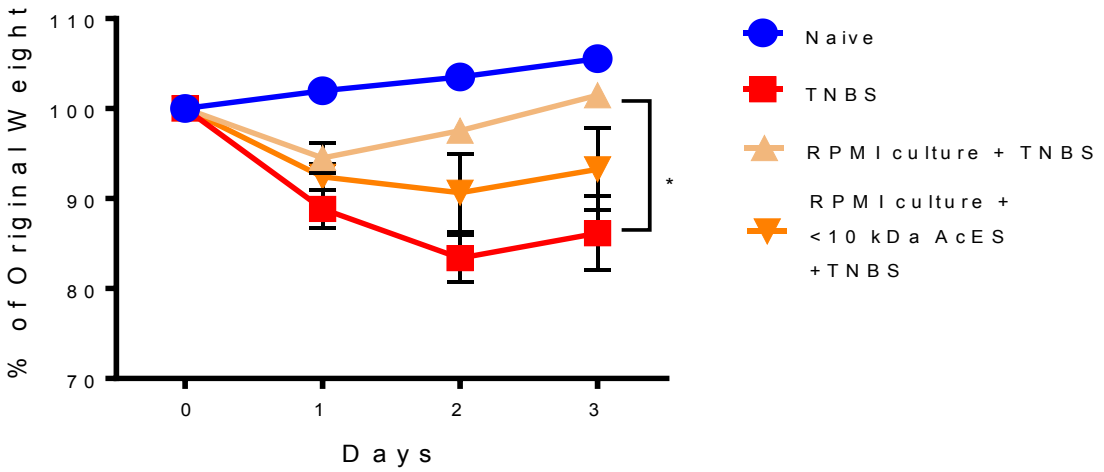


Figure 4.5. Weight loss induced by TNBS chemical colitis in C57BL/6 mice. The administering 50ug/mouse of RPMI culture media (prepared in the same manner as LES) afforded significant protection (* P=0.048) against TNBS-induced weight loss when compared to TNBS-treated controls. In contrast mice that are pre-treated with 50ug/mouse of LES from hookworms cultured in RPMI show no appreciable protection when compared with the TNBS negative controls Significance was determined using 1-way ANOVA (n=6)

These results indicate that RPMI is unsuitable as a media for *A. caninum* culture when the focus is on small molecule anti-inflammatory activity. This screen also highlights the necessity to validate the media used in further work in the TNBS colitis model to ensure that it is not a contributing factor to protection.

4.4 Identifying a new culture method for ES production for small molecule study

To date, it has not been possible to culture hookworms through all their life stages *in vitro*. This is because we do not fully understand the cues this parasite requires from the host to trigger life stage changes during its complicated lifecycle. To facilitate my study, it was necessary to harvest adult hookworms directly from the intestine of the host animal and culture these organisms *ex vivo*.

There has been an evolution of culture methods for the dog hookworm as the nature of enquiry has changed. These can grouped into three broad themes: (i) physiological studies; (ii) proteomics studies; and (iii) my current interest in small molecule secreteomics and metabolomics. This study found itself at the crux of the proteomic/small molecule

transition, so a number of depleted media combinations were assessed for their ability to support hookworms in culture with a view to subsequent small molecule isolation and *in vivo* testing in a colitis mouse model. The remainder of this chapter will focus on substrate selection and the ongoing experimental workflow.

Physiological studies of *A. caninum* are aimed at optimising the fecundity versus the longevity of the parasite in culture. This is determined by both the time the organism survived *in vitro* and the yield of egg production/shedding by female worms. The use of Krebs-ringer solution supplemented with 50% dog serum resulted in hookworm survival *in vitro* for >10 weeks [61]. This culture method has been shown to be suitable for studying basic physiology of the organism, as well as the effectiveness of anti-helminthic therapy *in vitro*. The presence of dog serum however precluded analyses of the protein and small molecule content of AcES. Worms were instead cultured in PBS, but rapidly became immotile and were lethargic by 12 hours and up 60% dead by 22 hours [396]. RMPI and Glutamax™ were later tested [5, 161] and supported hookworms for up to three days in culture.

It was decided that three culture conditions would be trialled for their ability to support adult hookworms *in vitro* (Table 4.1). The specific aim of these formulations was to reduce possible small molecule confounders while supplying an energy source; D-glucose as a carbohydrate source and Glutamax™ as an amino acid source. Glutamax™ is a dimer of glutamine and alanine. Concentrations were selected based on those provided in RMPI media formulation (Table 4.1.). Culturing worms in PBS (Table 4.1) was also conducted for comparative purposes.

Culture media formulations	Key ingredients of culture conditions for *A. caninum* adults	
1% Glutamax	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (Gibco, Life Technologies)	485 mL
	2% antimycotic/antibiotic	10 ml anti/anti
	1% Glutamax.	5 ml Glutamax
2g/L Glucose/ 1% Glutamax	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (Gibco, Life Technologies)	490 mL
	2% antimycotic/antibiotic	10 ml anti/anti
	1% Glutamax 2g/L glucose	5 ml Glutamax 1 g D-glucose
2g/L Glucose	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (Gibco, Life Technologies)	490 mL
	2% antimycotic/antibiotic	10 ml anti/anti
	2g/L glucose	1 g D-glucose
PBS†	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (Gibco, Life Technologies)	490 mL
	2% antimycotic/antibiotic	10 ml anti/anti
RPMI†	RPMI 1640 (Gibco, Life Technologies)	485 mL
	2% antimycotic/ antibiotic	10 ml anti/anti
	1% Glutamax.	5 ml Glutamax

Table 4.1. Culture conditions employed in this study for the aim of harvesting low molecular weight ES from *A. caninum*. †Both RPMI [5, 161] and PBS [397] formulations are included in this table for comparison and were employed in the initial stages of this study.

4.5 Results

Adult *A. caninum* were collected as described in Chapter 3 and cultured for a total of three days and survival recorded. The percent survival of adult hookworms is reported (Figure 4.7). Based on these results any of the three culture formulations was deemed appropriate for culture of *A. caninum* ES and are comparable to the established RPMI culture method. In all three culture conditions, hookworms were noted to shed ova across the course of the experiment. The PBS only culture method showed poor survival and hookworms surviving past 24hours were lethargic and many only responded when gently palpated.

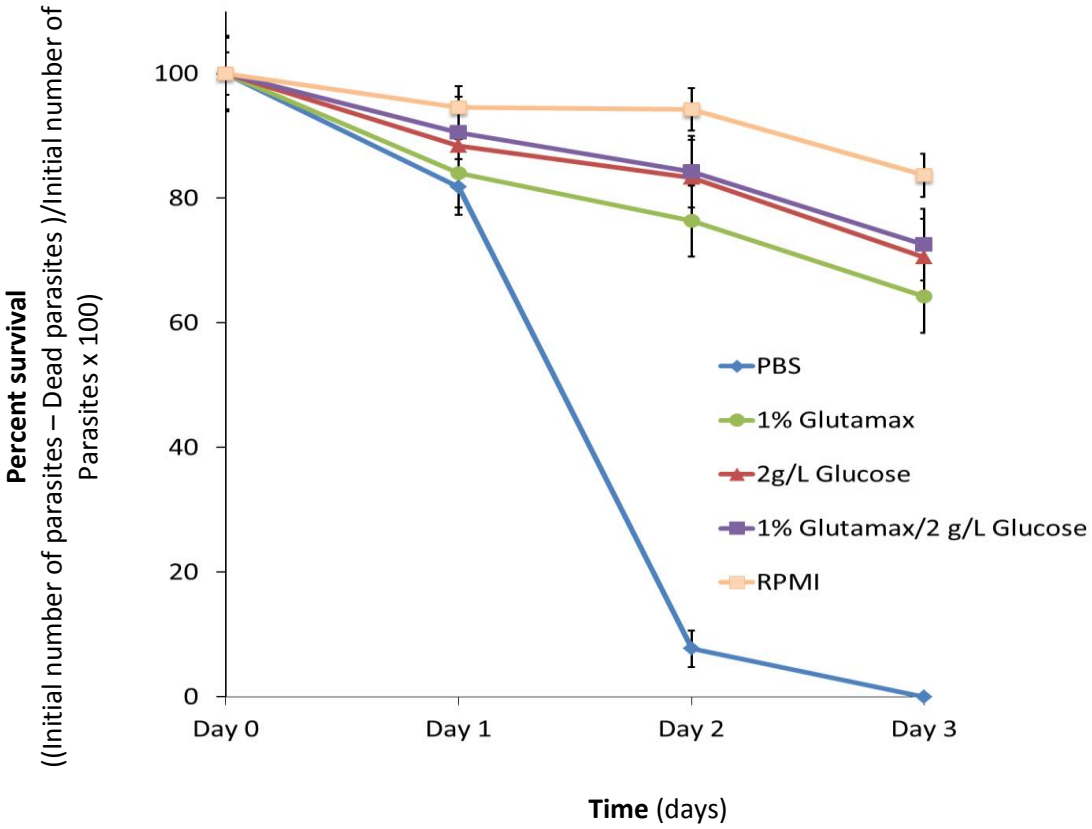


Figure 4.7. The percent survival of adult *A. caninum* in different media formulations. Hookworms cultured in PBS showed poor survival over the course of monitoring. Worms supplemented with the other formulations showed comparable survival rates to RPMI.

4.5 Experimental Rationale and Workflow

The general experimental workflow that formed the basis for this thesis is detailed in Figure 4.8. Using this workflow, it was possible to concertina the dual aims of molecular characterisation of LES and identification of anti-inflammatory bioactivity.

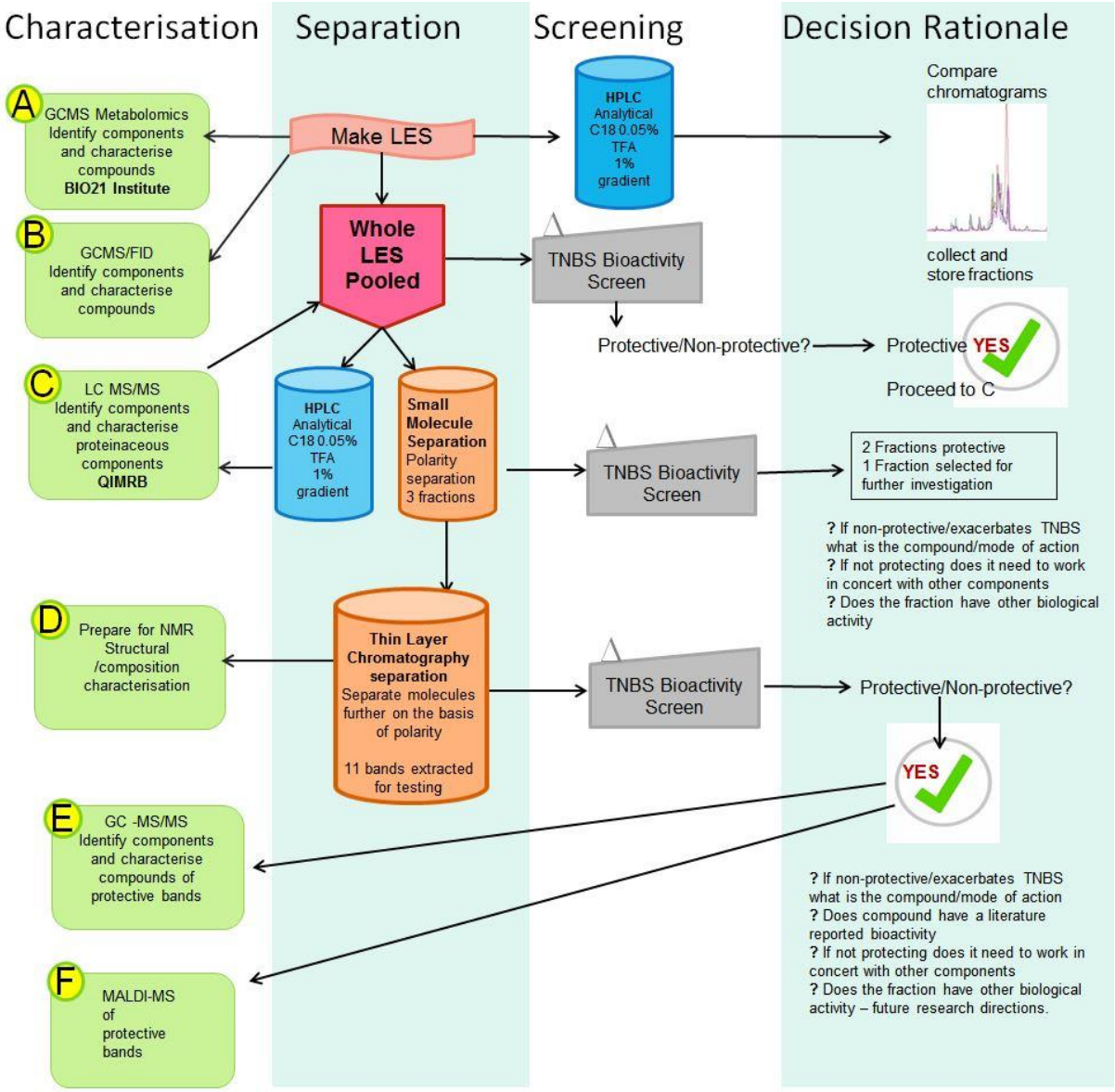


Figure 4.8 Workflow of *A. caninum* LES characterisation, separation, and *in vivo* screening for anti-colitic bioactivity along with the decision rationale for each step.

4.6 Discussion

The aim of this body of work is to identify immunomodulatory small molecules produced by the dog hookworm at the gut interface. Since LES was harvested from the diffusate using a 10 kDa size exclusion column a wide range of molecules are possible. This work is builds upon on the initial screening of RPMI derived LES was carried out by Markus Baumann and detailed in his Master's thesis [373]. His work demonstrated that while LES showed little protection against TNBS induced colitis, some of the HPLC-separated fractions resulted in protection against TNBS-induced weight loss and macroscopic pathology when compared to control mice that received TNBS. Based on 1-D NMR it was concluded that the protective

component of low molecular weight AcES was proteinaceous in nature [373]. Work here has failed to support this conclusion.

It was not possible to establish a pool of peptides for screening here. On reflection, this may be due to collection methods. Culture media is only collected every 24 hours. Peptides unless they are stabilised in some way (through cyclisation or multiple disulphide bonds) are unstable. Most peptides are thought to have a half-life of between 2-30 minutes *in vivo* in part due to proteolysis. Since we know that there is at least six proteases identified in the ES of *A. caninum* [161] prolonged culture is likely to limit the ability to detect peptides due to degradation. Due to limited access to source material it was decided to shift focus to the small molecules that *A. caninum* produces and look for molecules that may have anti-inflammatory potential.

I was able to show here that mice treated with RPMI alone are protected against the effects of TNBS induced weight loss. This may be due to one or many of the components of RPMI and has been discussed in more detail in Chapter 2. The contribution of the media indicator phenol red was also problematic as it had an overwhelming spectral presence and co-eluted with fractions of interest. The technical difficulties of the small molecules present in the RPMI meant that other formulations had to be considered. I have screened a number of simple formulations here that are able to support hookworms in culture for up to three days. These depleted media mean that hookworms may not be cultured in optimal conditions to support physiological functions yet all three formulations displayed ova shed indicating that fecundity was maintained.

In the shift from proteomic studies to small molecule studies has shown that established culture methods for proteomics are not suitable. The study of helminth small molecules means that a complex media adds a technical difficulty that can be overcome by the use of minimal media combinations.

The next chapter: In the next chapter I utilise small molecule techniques to separate small molecule components from LES. This is guided by the TNBS chemical colitis screen (a broad based bioactivity screen for anti-inflammatory potential). Using these combined techniques I highlight fractions from LES that have therapeutic potential.

Chapter 5

Hookworm LES protects against TNBS-induced acute colitis in mice

Preamble

In this chapter I present the results of screening *A. caninum* LES produced in a variety of media combinations using a mouse model of TNBS chemical colitis.

I demonstrate that protection can be elicited with LES collected from hookworms cultured in minimal media supplemented with Glutamax™, but none of the other media combinations investigated. I also demonstrate that the media alone has no protective properties in the TNBS colitis model, indicating that it is the hookworm derived molecules responsible for protection.

I show that hookworm LES components can be separated by polarity extraction and bioactivity is retained in some fractions.

Finally, a protective fraction is further separated by thin layer chromatography into 11 fractions. Only four of these fractions elicit protection in the TNBS colitis model, demonstrating that protection is caused by distinct moieties within hookworm LES.

Outputs from this chapter

The low molecular weight secretome of *Ancylostoma caninum* protects against colitis in a murine model. Shepherd, C., Wangchuk, P., Constantinoiu C., Navarro S., Wilson D., Kouremenos, K. A., De Souza, D. P., McConville, M., Daly N., and A. Loukas

First Author

IN DRAFT; Target journal: International Journal for Parasitology

5.1 The TNBS chemical colitis model as a bioactivity screen

The murine TNBS chemical colitis model used in this thesis is adapted from the method described by Ruyssers *et al.* [5] and Wirtz *et al.* [6]. In this model, trinitrobenzene sulfonic acid (TNBS) is administered directly to the colon via rectal enema of the animal in an ethanol solution. The ethanol acts to disrupt the cells of the gut lining, lyse bacteria, as well as react with gut contents. The highly reactive TNBS then haptenises these normally harmless components, allowing them to translocate and be presented to the immune system, initiating a series of inflammatory responses. Outwardly, the mouse will present as if they have an acute bout of UC. They will lose weight over the course of a couple of days, be lethargic, lose interest in preening and their natural curiosity will wane. All of these changes can be closely measured to gain clinical indices scores. By the third day the TNBS/Ethanol treated mice will start to recover, and it is here that our screening ends.

The TNBS colitis model is mechanistically complex, and triggers Th1, Th17 and Th2 immune responses, as well as inducing innate responses, including the inflammasome pathway. It is for this precise reason that this model lends itself well to screening for LES molecules with unknown targets/mechanisms of action. At the initial screening stage, assessment of cytokine responses confirms that the immune response is stimulated as part of this protection process. As the separation of LES components progresses, it is possible to determine whether this protection is due to individual molecules or groups of molecules acting synergistically. By assessing mouse cytokine responses, we may be guided to select appropriate and more refined mechanistic models for future testing. It is worthwhile emphasising here that the TNBS chemical colitis screen is used in this body of work in its capacity as a screening tool, and not as a final mechanistic model.

Additional advantages of the TNBS colitis model for this study are that it uses relatively small amounts of test material (single treatment per mouse) and the experiment has a short timeframe. This means valuable material can be used sparingly and that protection is gauged quickly by the assessment of weight loss and other clinical and pathologic indices. This allows successive screens to occur in a relatively short period.

The following data is presented in the context of TNBS chemical colitis as a bioactivity screen for protection against colitis. I combine this bioactivity screen with small molecule

techniques for the separation of LES components, with the ultimate aim of isolating hookworm molecules that may have therapeutic potential. The experimental rationale for proceeding to the next step is outlined throughout this chapter.

5.2 Hookworms cultured in Glutamax™ media produce LES components that protect against TNBS colitis

Four media combinations were trialled for the culture of *A. caninum* and harvesting of small molecule ES. These are outlined in Chapter 4 but are briefly; PBS only, PBS with 1% Glutamax™, PBS with 1% Glutamax™ combined with 2 g/L D-glucose and finally PBS with 2 g/L D-glucose. All of these media contained Anti-Anti (Gibco) to suppress fungal and bacterial growth during culture. Samples were prepared as outlined in Chapter 3 and administered to mice i.p. at a dose of 50 μg/mouse prior to TNBS colitis induction. In Figure 5.1 weight loss of each treatment group as a percentage of their starting weight is plotted over the course of the experiment. Only LES derived from hookworms cultured in Glutamax™ protects against weight loss.

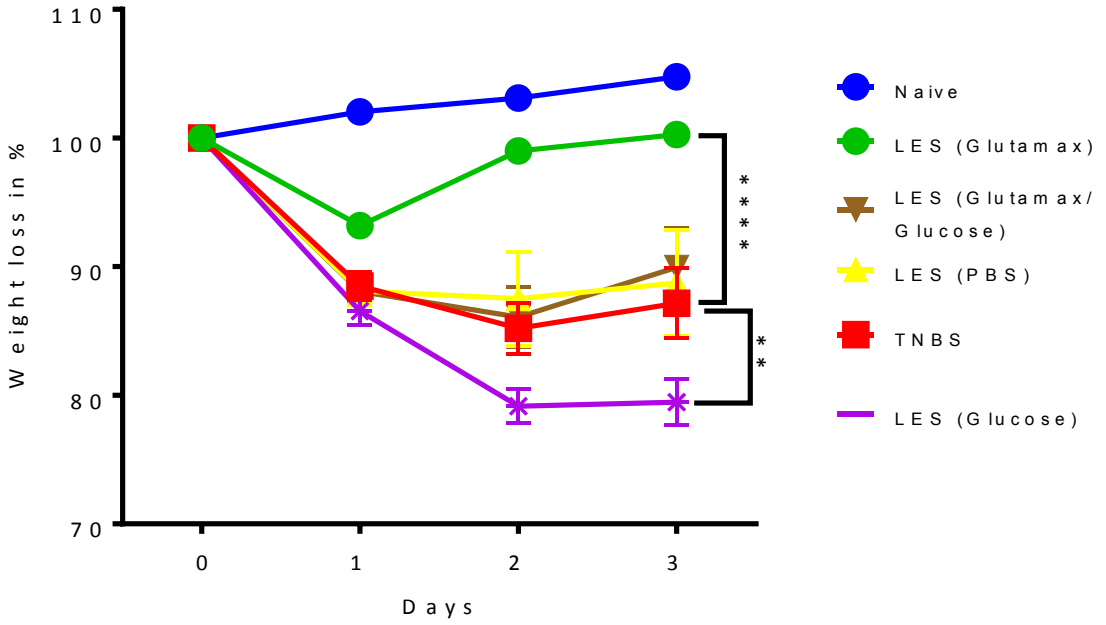


Figure 5.1. Mice treated with LES harvested from adult *A. caninum* cultured in 1% Glutamax™ are significantly protected against TNBS induced weight loss. Weight loss of mice in the treatment groups is compared to the TNBS control by 2-way ANOVA. Mice treated with 50 µg of LES material harvested from hookworms cultured in 1% Glutamax™ showed significant protection against weight loss when compared to the TNBS control (**** = P<0.0001). Hookworms cultured in either PBS (LES (PBS)) alone or a combination of 1% Glutamax™/2 g/L D-glucose (LES (Glutamax™/Glucose) showed no significant difference in weight change from the TNBS control. Finally, hookworms cultured in a medium of 2 g/L of D-glucose had significantly lower weights than the TNBS control group (** = P<0.01). All results are the aggregation of at least two independent experiments. Treatment groups received 50 µg of LES material per mouse via i.p. injection (n=12).

Interestingly, the LES (Glucose)-treated mice fared worse than negative control mice that received TNBS in this model. The LES (Glucose)-treated mice lost significantly more weight than TNBS controls and did not recover in the timeframe of the experiment. It would be interesting to explore the drivers of this observation later.

Colon lengths of treated and control mice were assessed for shortening, one of the key indicators of TNBS chemical colitis. Colon lengths were measured at the end of the experiment upon euthanasia of the mice. Mice pre-treated with 50µg of LES from hookworms cultured in 1% Glutamax™ had significantly longer colons than the TNBS controls.

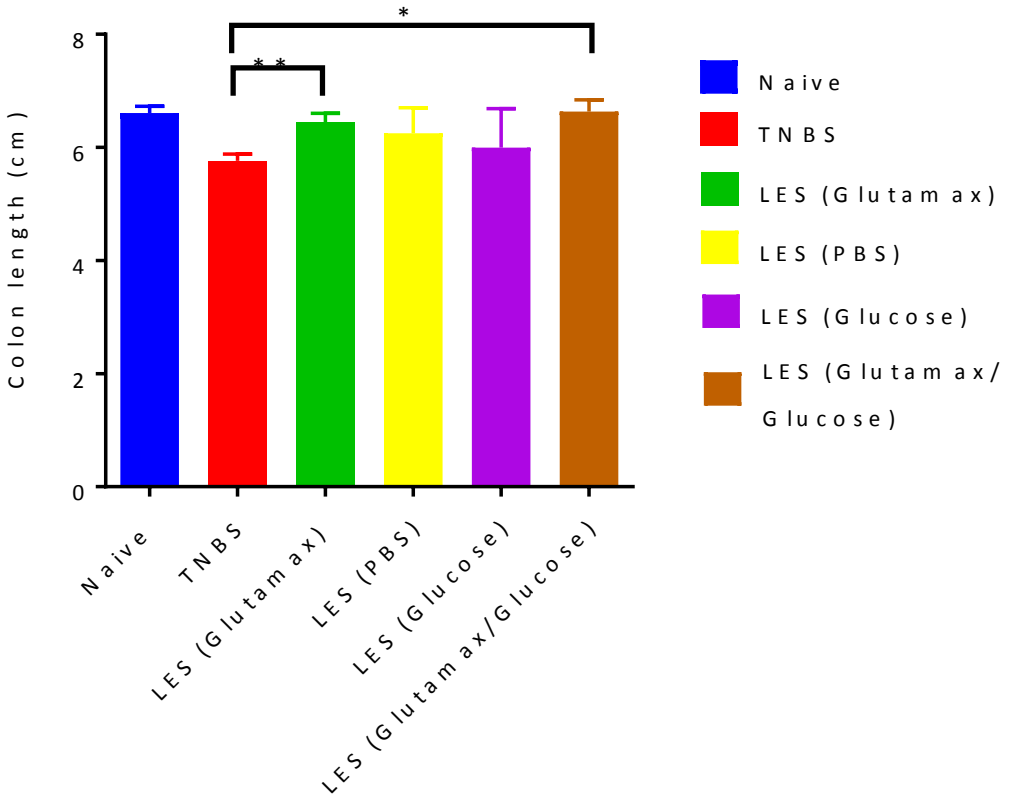


Figure 5.2. Mice treated with LES harvested from adult *A. caninum* cultured in 1% Glutamax™ are significantly protected against TNBS-induced colon shortening. Colon length of mice in treatment groups is compared to the TNBS control by 2-way ANOVA. Mice treated with 50 µg of LES material harvested from hookworms cultured in 1% Glutamax™ showed significant protection against colon shortening when compared to the TNBS control (** P = 0.018). Colon lengths of mice treated with LES from worms cultured in PBS (LES (PBS)) or 2 g/L of D-glucose (LES (Glucose)) showed no significant difference from the TNBS control group. Colon lengths of mice treated with LES from hookworms cultured in a combination of 1% Glutamax™/2 g/L D-glucose (LES (Glutamax™/Glucose)) showed significantly longer colons than the TNBS control group (* P =0.0197). All results are the aggregation of at least two independent experiments. Treatment groups received 50 µg of LES material per mouse via i.p. injection (n=12).

These two results clearly indicate that Glutamax™ cultured hookworms produce small molecules that are able to protect against TNBS chemical colitis. Due to the complications of media selection it was considered prudent to screen Glutamax™ media (PBS, 1% Glutamax™ and 2X Anti-anti (Gibco)) in the TNBS model to eliminate the possible role of media ingredients in protection against TNBS colitis. Glutamine is one of the components of Glutamax™. Glutamine is considered an immune supportive nutrient [390], and administration has been shown to be protective in cases of colitis in humans [385, 398].

Administration of glutamine results in reduction of TNF and the main pro-inflammatory cytokines released in in active CD [385]. I also addressed the role of Anti/Anti (Gibco) in protection. There are no reported anti-inflammatory activities for either streptomycin or penicillin and its breakdown products (penilloic acid, penicilloic acid and isopenillic acid), however the anti-mycotic amphotericin B has reported immunomodulatory activity in the literature [399]. The results for this screen are detailed in Figure 5.3.

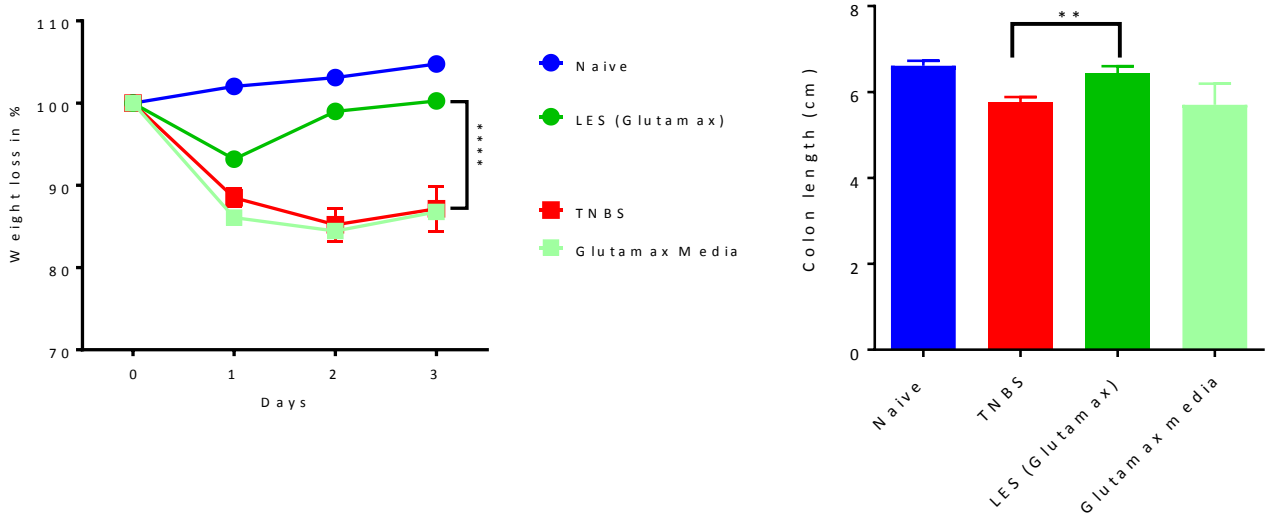


Figure 5.3. Mice treated with 1% Glutamax™ media alone are not protected against TNBS-induced weight loss and colon shortening. Weight loss (left) and colon lengths (right) of mice treated with Glutamax™ media alone were comparable to the TNBS control, and conferred no protection compared to LES (Glutamax)-treated mice ($P<0.0001$ weight loss; $P<0.01$ colon length). Weight loss and colon lengths were assessed by 1-way ANOVA. Mice were treated with 50 µg material via i.p. injection. All results are the aggregation of at least two independent experiments. Treatment groups, n=8.

Based on the results it was decided that *A. caninum* would be cultured in 1% Glutamax™ media for all further work. These preparations showed convincing protection in the TNBS colitis model and the work that follows details the separation of LES (Glutamax™) into fractions in an attempt to isolate molecules that exhibit anti-inflammatory activity but also have therapeutic potential (or druggability). Characterisation of *A. caninum* LES can be found in Chapter 6.

5.3 *A. caninum* LES (Glutamax™) treatment results in improved clinical indices, reduced histopathology and promotes anti-inflammatory cytokine production in TNBS colitis

Given the significant protection against TNBS-induced weight loss and colon shortening afforded by LES (Glutamax™), the nature of the protection was examined in closer detail. Clinical indicators, macroscopic pathology scores, histology, and cytokine analyses were carried out. Overall mice that were pre-treated with LES (Glutamax™) were much healthier and showed less tissue damage than TNBS alone treated mice. In addition, treatment resulted in altered levels of cytokines associated with IBD. Figure 5.4 shows the clinical indicators of mouse health (mobility, piloerection, stool quality/output) along with the clinical score (an aggregated score including weight loss indices). In all cases LES (Glutamax™)-treated mice are significantly healthier than the TNBS control mice during the course of the experiment.

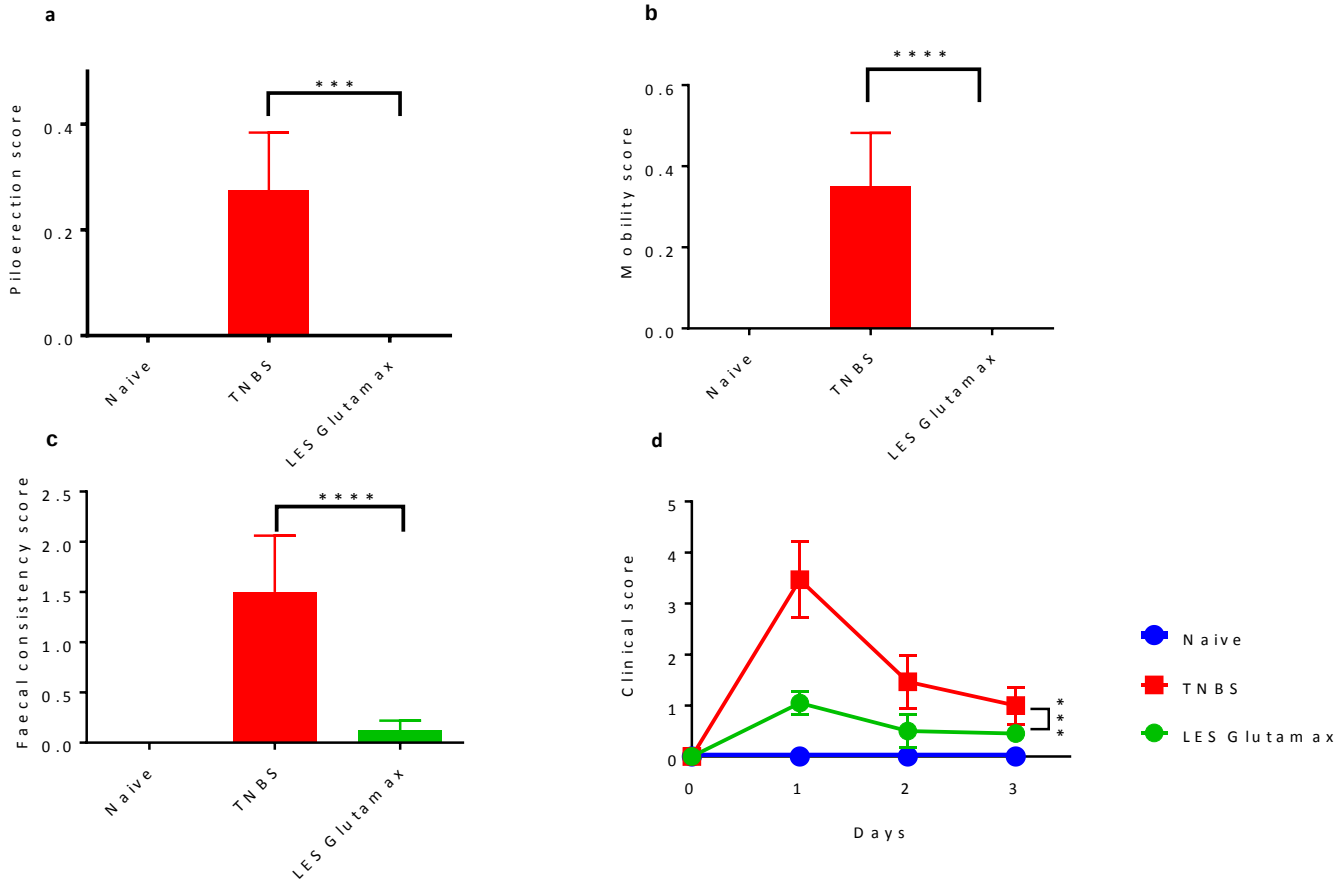


Figure 5.4. Mice treated with 50 µg of LES (Glutamax™) have significantly lower clinical disease scores than the TNBS only treated controls. Piloerection score for mice treated with LES (Glutamax™) is significantly lower than the TNBS control group (a; *** P = 0.0003). The mobility score was significantly lower for the LES (Glutamax™) treatment group when compared to the TNBS control (b; **** P <0.0001). Similarly, stool consistency showed a marked improvement in LES (Glutamax™)-treated mice when compared to TNBS controls (c; **** P <0.0001). Finally, there was a significant difference in clinical scores of LES (Glutamax™)-treated mice compared to TNBS controls (d; *** P=0.0005). All differences were assessed by 2-way ANOVA when compared to the TNBS control. All results are the aggregation of at least two independent experiments. Treatment groups received 50 µg material per mouse via i.p. injection (n=10).

As part of the TNBS colitis, colon quality at necropsy can be assessed. Markers of colitis such as colon edema and thickening, adhesion to tissues and the presence of ulcers are macroscopic indicators of the colitic process. In Figure 5.5 I compare the colons of LES (Glutamax™)-treated mice with the TNBS control; these mice have significantly lower macroscopic indicators of colitis than the negative controls.

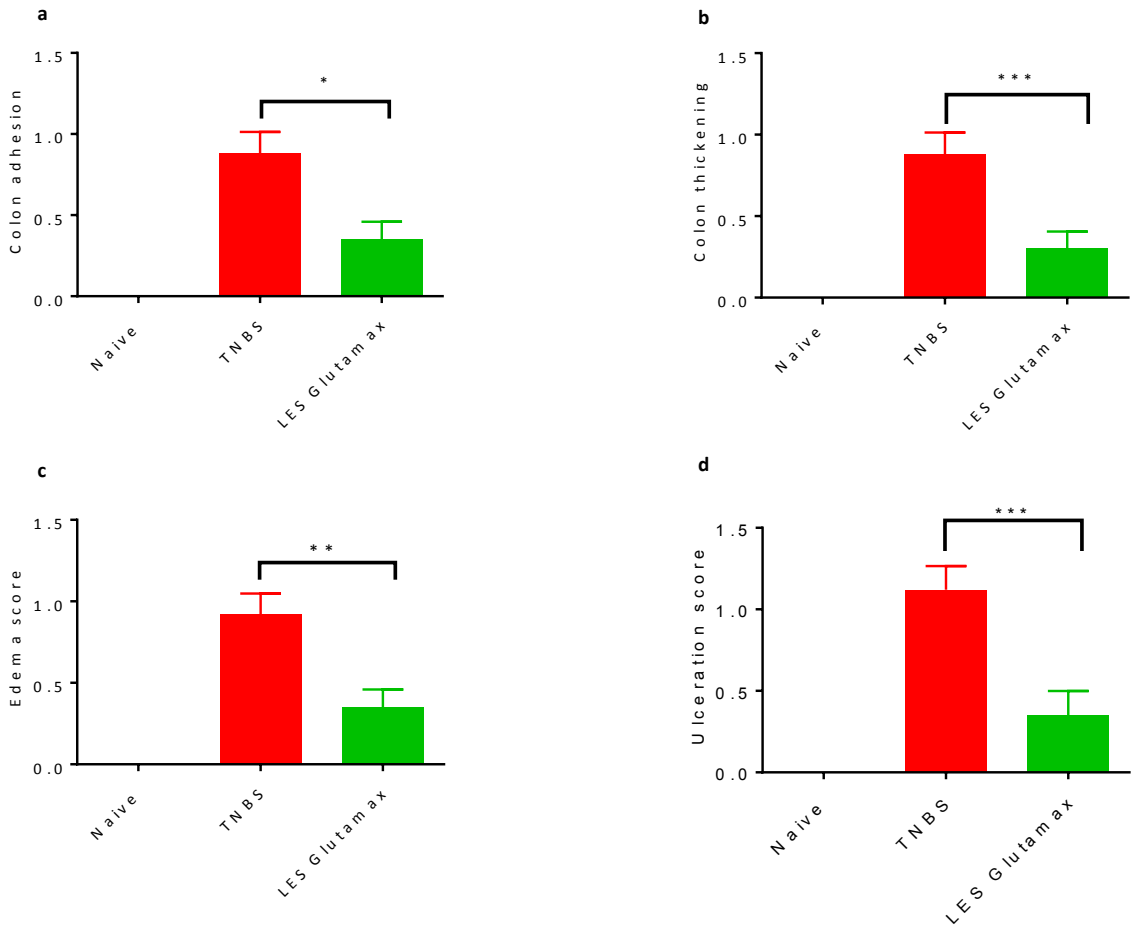


Figure 5.5. Mice treated with 50 µg of LES (Glutamax™) prior to administration of TNBS have significantly lower macroscopic scores than controls. Colon adhesion for mice in the LES (Glutamax™)-treated group is significantly lower than the TNBS control group (a; * P = 0.0111). Colon thickening was reduced in the LES (Glutamax™)-treated group when compared to the TNBS control (b; *** P = 0.0002). Similarly, when assessing edema there was an improvement in LES (Glutamax™)-treated mice when compared to TNBS controls (c; ** P = 0.0024). Finally, ulceration is significantly lower in LES (Glutamax™)-treated mice compared to TNBS controls (d; *** P=0.0001). All differences were assessed by 1-way ANOVA when compared to the TNBS control. All results are the aggregation of at least two independent experiments. Treatment groups received 50 µg material per mouse via i.p. injection (n=10).

Colon tissue was stained with haematoxylin/eosin (H&E) stain and a blind scoring was carried out of on the criteria outlined in Chapter 3 (Material and Methods). Briefly, slides were assessed at high resolution for ulceration, infiltration of the muscularis mucosa, overall integrity of the epithelium and the presence of lymphoid follicles, and scored to a maximum 16. Figure 5.6 shows typical representations of histology of naïve, TNBS only, and mice pre-

treated with LES (Glutamax™) prior to TNBS, as well as the aggregate histological score for each treatment group.

Colons of LES (Glutamax™)-treated mice were also assessed for production of a number of cytokines to confirm that treatment protected against changes in cytokine-driven pathological outcomes and resulted in an anti–inflammatory profile. These results were also used as a guide to frame the isolation of the immunomodulatory agents that may be present in LES (Glutamax™). Figure 5.7 presents cytokine results for both colon culture and colon homogenates. All cytokine results presented are normalised to tissue weight to allow for comparison across experimental groups and will be reported in pg cytokine/mg tissue.

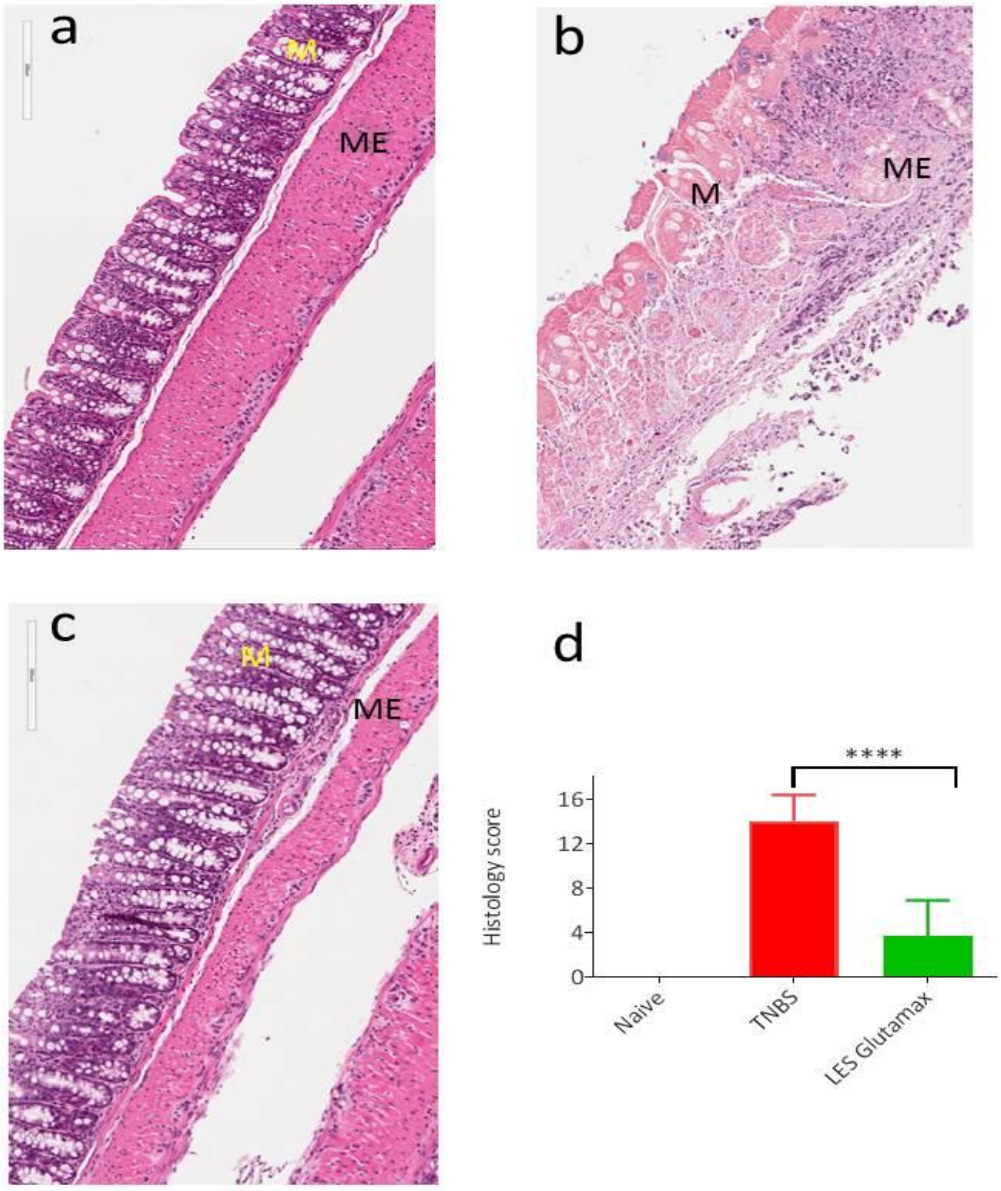


Figure 5.6. Pre-treatment with 50 µg of LES (Glutamax™) per mouse results in significant protection against TNBS-induced colitis. Panels a-c show longitudinal sections (3-4 µm) of mouse colon stained with haematoxylin & eosin; the white bar represents a scale of 200 µm. All pictures are in the same orientation. Panel a) shows a typical section from a naïve (no treatment mouse) colon. Panel b) shows a section from a TNBS-treated colon – note the massive destruction of mucosal layer (M), thickening, and infiltration of the muscularis externa (ME) due to ulceration. Panel c) shows colon tissue from mice pre-treated with 50 µg of LES (Glutamax™) via i.p. injection and then exposed to TNBS, note the maintenance of mucosal layers and relatively little immune infiltration of the tissue. Panel d) shows the aggregate of blinded scoring of histopathology slides, where a score of 0 is completely normal tissue and 16 completely degraded. TNBS treated mice score highly whereas LES (Glutamax™)-treated mice have significant (1 way ANOVA n=8) protection against colitis (**** $P<0.0001$). It should be noted that LES (Glutamax™)-treated mice only received a score above zero based on lymphoid follicles.

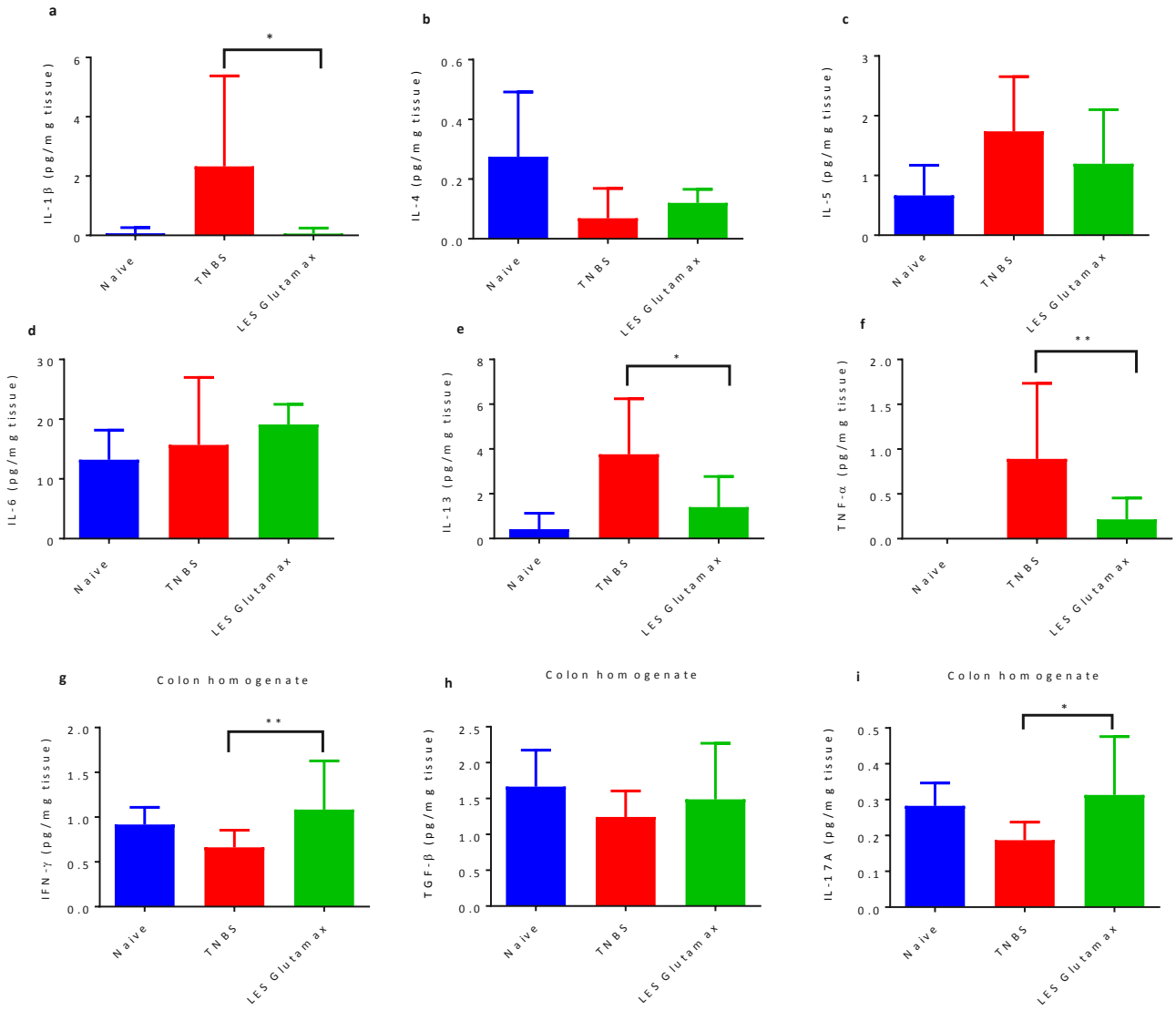


Figure 5.7. Changes in colon cytokine levels induced by treatment with 50 µg LES (Glutamax™) prior to induction of TNBS colitis in mice. Of the nine cytokines assayed, five are significantly different between the treatment and control groups after TNBS administration (1 way ANOVA). Cytokines were measured from colonic tissue cultured for 24 hours in complete media (a-f). LES (Glutamax)-treated mice had significantly lower levels of a) IL-1β (* P = 0.0109); e) IL-13 (* P = 0.0100) and f) TNF (** P = 0.0090) when compared to TNBS control groups. There were no significant differences in the levels of IL-4 (b), IL-5 (c), and IL-6 (d). Measurement of cytokine levels in homogenates of colons from LES (Glutamax™)-treated mice showed a significant increase in IFN-γ (g; ** P = 0.0090) and IL-17A (i; * P = 0.0154) with respect to the TNBS control. There were no discernible changes in TGF-β levels (h). All results are the aggregation of at least two independent experiments. Treatment groups received 50 µg material per mouse via i.p. injection (n=8).

These results are notable for a number of reasons; cytokines from the colon cultures indicate an anti-inflammatory response induced by LES (Glutamax™) but colon homogenates have increased levels of pro-inflammatory cytokines like IL-17A and IFN-γ. There is also a mismatch of high IL-17A levels and those of the downstream cytokines of TNF and IL-6. What is perhaps most interesting is that a spectrum of Th1, Th2 and Th17 cytokines are affected by LES (Glutamax™) treatment. It is worth noting that IL-17A does not always display pro-inflammatory activity but also plays a role in anti-inflammatory/regulatory responses. This is the first time that the small molecule milieu produced by any helminth has been shown to induce protection against colitis and induce an anti-inflammatory response.

Encouraged by these results, I progressed with separation of LES (Glutamax™) utilising small molecule chemistry techniques. These techniques have been used extensively in the study of ethnobotanicals for the isolation and identification of small molecules. It was hoped that using these processes it would be possible to identify the anti-inflammatory agents present in hookworm LES (Glutamax™) with a view to exploiting their therapeutic potential.

5.4 Polarity extraction of LES (Glutamax™) into three fractions yields only two fractions that retain protection in the murine TNBS colitis model.

The first separation process was the Folch extraction [400] which primarily separates components on the basis of their polarity. Three fractions of LES (Glutamax™) were generated using this technique; the first was chloroform: methanol (CM) fraction; the second was the non-polar ethyl acetate (EA) fraction; and a final polar salts (PS) fraction was generated. There were still concerns regarding the concentration of media components at this stage, so a polarity extraction of media alone was also carried out and tested alongside the LES (Glutamax™) fractions. The three LES (Glutamax™) fractions were then tested in TNBS colitis.

At the same time as these experiments were carried out, I conducted a dosing study in the TNBS colitis model. Initial screens of the three LES (Glutamax™) fractions in TNBS colitis were carried out using 50 μg of material per mouse, but this did not significant protection against colitis. Given that the extraction processes would have concentrated some

components (including potentially toxic ones); I tested a lower dose (20 µg) of material in TNBS colitis.

When compared to the TNBS control group, mice treated with both the EA and CM LES fractions showed significant protection against TNBS-induced weight loss (Figure 8a). The PS LES fraction elicited no significant protection in this model. To rule out the anti-inflammatory potential of Glutamax™ media components, the media alone (in the absence of hookworm culture) was extracted in the same manner as the LES (Glutamax™) material, and the EA and CM fractions were tested in the TNBS colitis model (Figure 5.8b). In this screen I demonstrated that protection was elicited by the EA media fraction but not the CM fraction. This fraction contained the most non-polar compounds, and were likely to be less soluble and therefore less bioavailable than compounds in the other fractions [24]. Moreover, it was decided that due to the confounding issue of media components present in the EA fraction that elicit protection against TNBS colitis, that the LES EA Glutamax™ fraction was not suitable for further exploration. I therefore decided to pursue the CM LES fraction for further experimentation. Screens of the media fractions - EA Glutamax™ and CM Glutamax™ - indicated that the EA fraction protected against weight loss (Figure 5.8b).

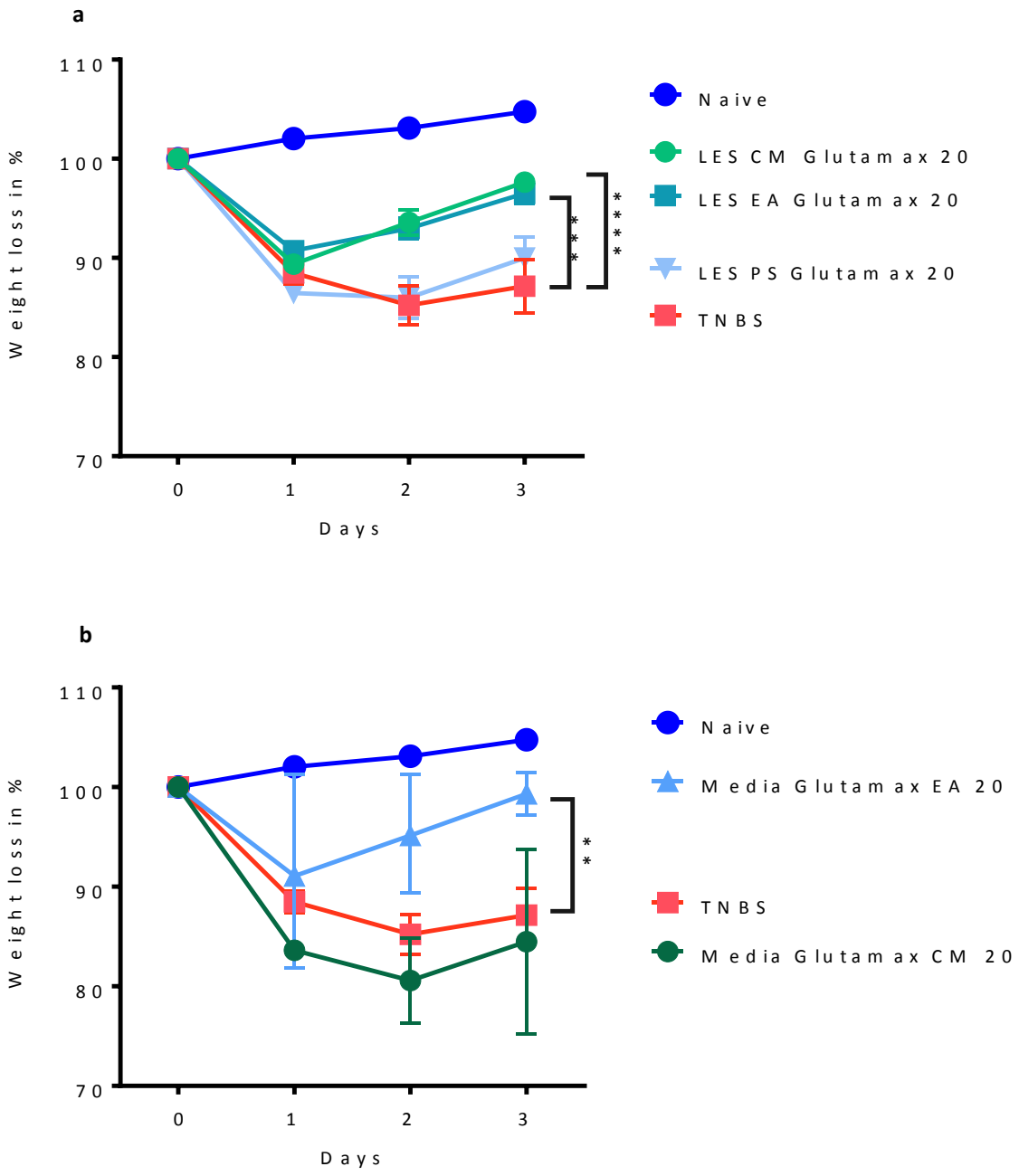


Figure 5.8. Polarity extraction of small molecule ES (LES) harvested from adult *A. caninum* cultured in 1% Glutamax™ yields two protective fractions. a) Mice treated with 20 µg of either EA LES Glutamax™ (*** P = 0.0001) or CM LES Glutamax™ (**** P<0.0001) showed significant protection when compared to the TNBS control (1 way ANOVA, n=10). Polar salt extracts elicited no protection in the TNBS colitis model. When mice were treated with 20 µg of the media via i.p. injection prior to TNBS administration, only EA Glutamax™ and CM Glutamax™ extracts elicited significant protection (** P = 0.0012) in the TNBS colitis model (1 way ANOVA, n=6).

A more thorough assessment of the anti-colitic properties of LES-CM was conducted to assess clinical scoring, histology and cytokine analyses.

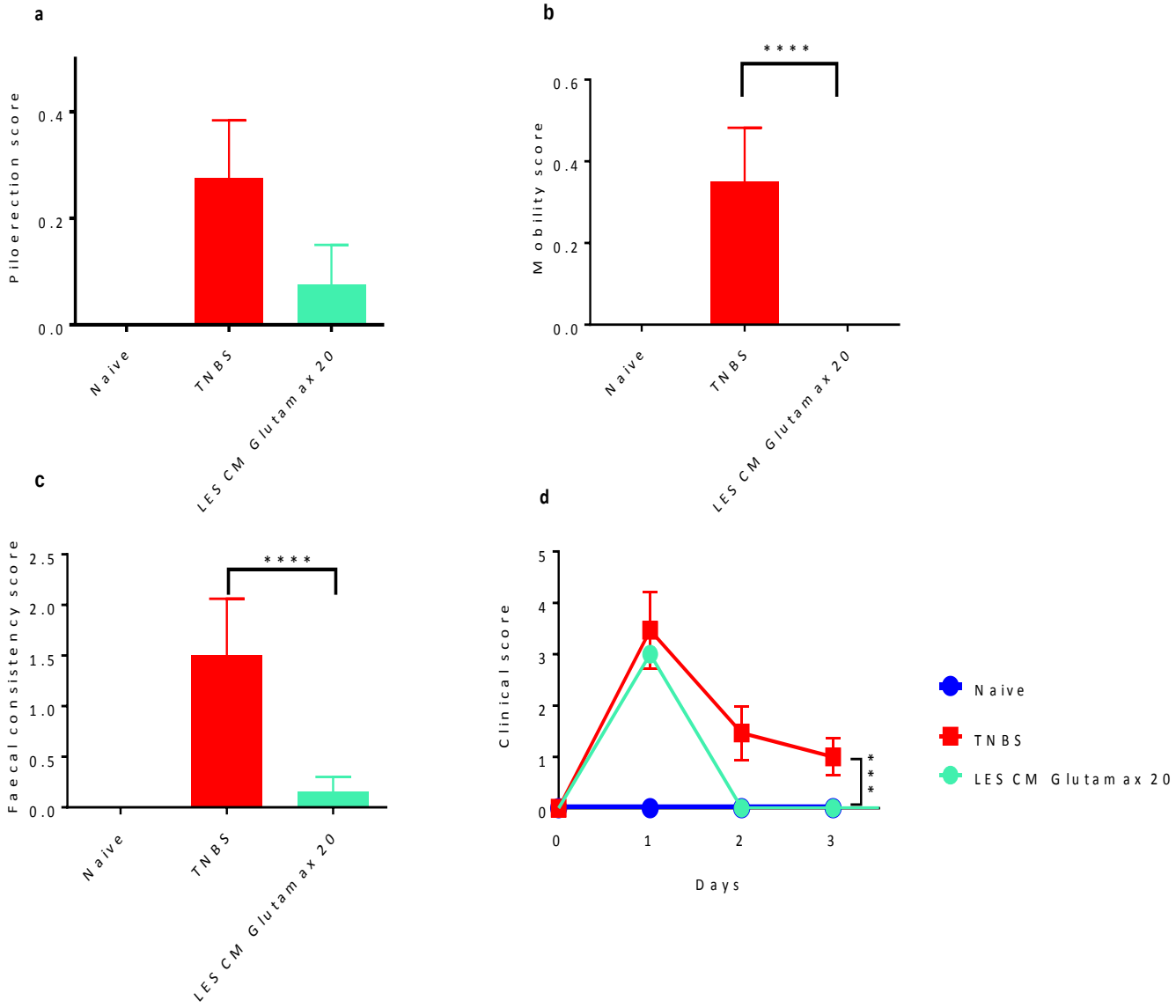


Figure 5.9. Mice treated with 20µg of LES CM (Glutamax™) have significantly lower clinical disease scores than the TNBS only treated controls. Piloerection score for mice treated with LES (Glutamax™) is not significantly lower than the TNBS control group (a). The mobility score was significantly lower for the LES (Glutamax™) treatment group when compared to the TNBS control (b; **** P <0.0001). Similarly, stool consistency showed a marked improvement in LES (Glutamax™)-treated mice when compared to TNBS controls (c; **** P <0.0001). Finally, there was a significant difference in clinical scores of LES (Glutamax™)-treated mice compared to TNBS controls (d; *** P= P=0.0042). All differences were assessed by 1-way ANOVA when compared to the TNBS control. All results are the aggregation of at least two independent experiments. Treatment groups received 20 µg material per mouse via i.p. injection (n=10).

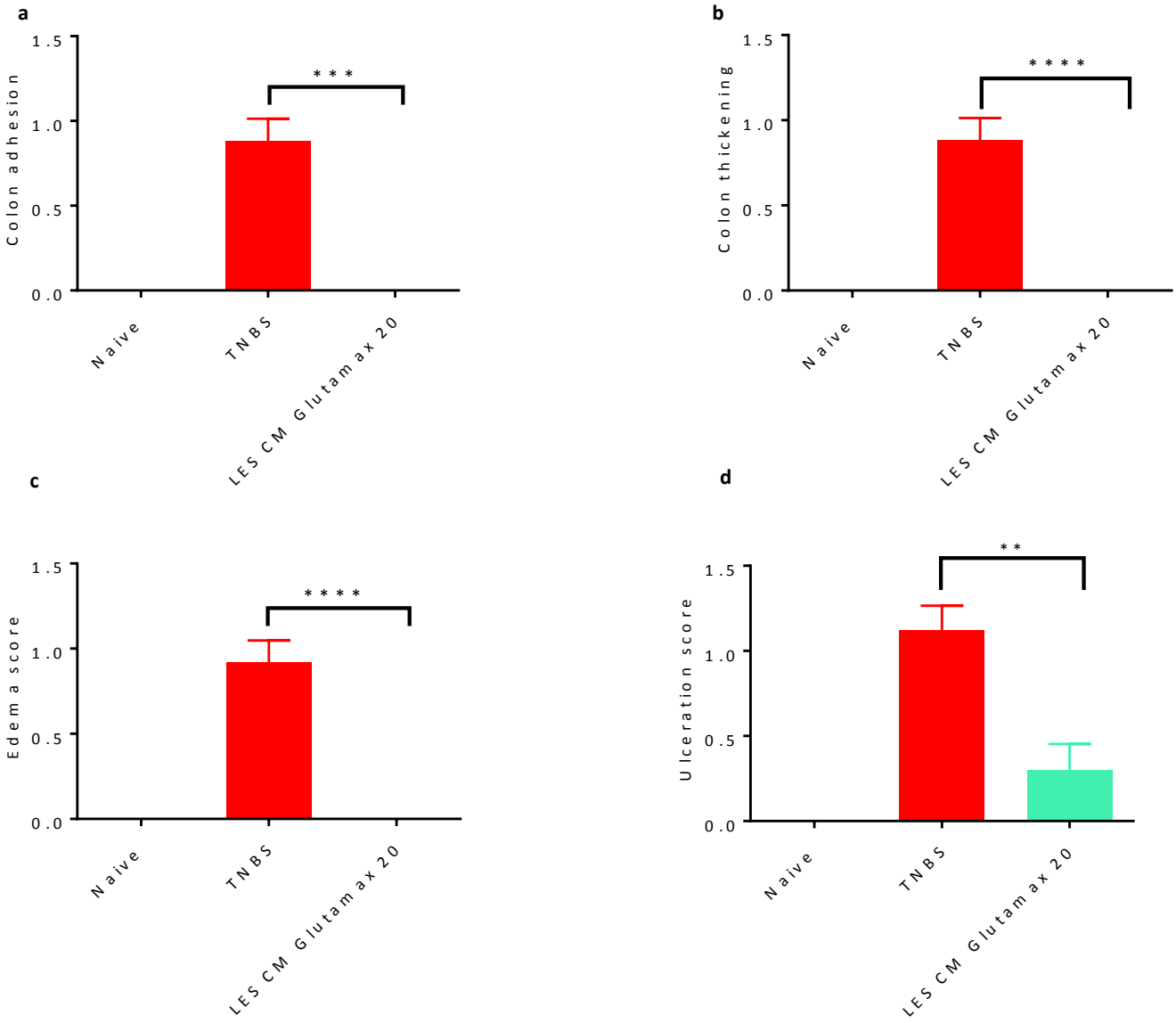


Figure 5.10. Mice treated with 20 µg of LES CM (Glutamax™) prior to administration of TNBS have significantly lower macroscopic scores than controls. Colon adhesion for mice in the LES CM (Glutamax™)-treated group is significantly lower than the TNBS control group (a; (*** P = 0.0002). Colon thickening was reduced in the LES CM (Glutamax™)-treated group when compared to the TNBS control (b; **** P <0.0001). Similarly, when assessing edema there was an improvement in LES CM (Glutamax™)-treated mice when compared to TNBS controls (c; (**** P <0.0001). Finally, ulceration is significantly lower in LES (Glutamax™)-treated mice compared to TNBS controls (d; ** P=0.0015). All differences were assessed by 1-way ANOVA when compared to the TNBS control. All results are the aggregation of at least two independent experiments. Treatment groups received 20 µg material per mouse via i.p. injection (n=10).

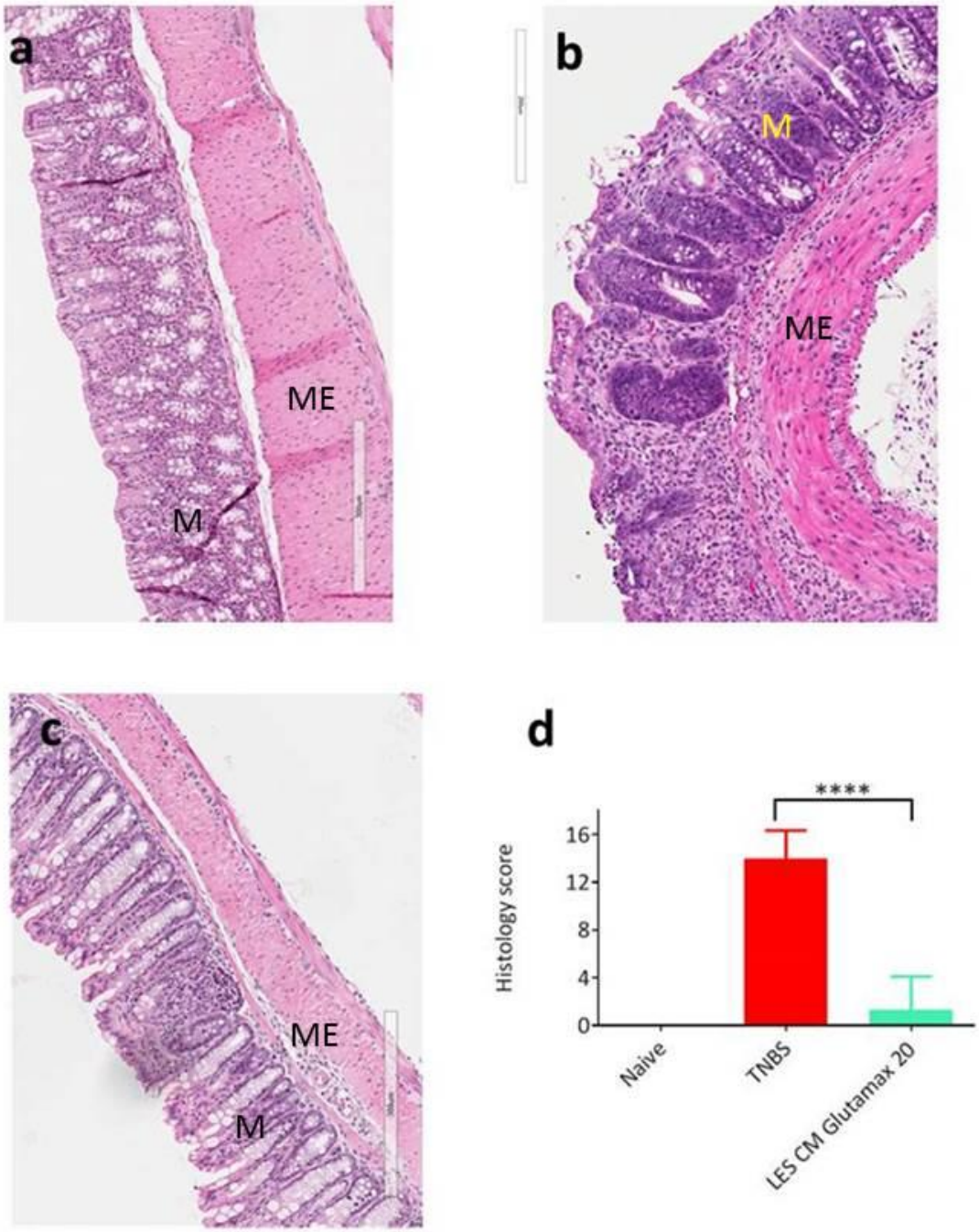


Figure 5.11. Pre-treatment with 20µg of LES CM (Glutamax™) per mouse results in significant but not complete protection against TNBS induced colitis. Panels a-c show longitudinal sections (3-4 µm) of mouse colon stained with haematoxylin & eosin; the white bar represents a scale of 200 µm. All pictures are in the same orientation. Panel a) shows a typical section from a naïve (no treatment mouse) colon. Panel b) shows a section from a

TNBS-treated colon – note the massive destruction of mucosal layer (M), thickening, and infiltration of the muscularis externa (ME) due to ulceration. Panel c) shows colon tissue from mice pre-treated with 20 μg of LES CM (Glutamax™) via i.p. injection and then exposed to TNBS, note the maintenance of mucosal layers and relatively little immune infiltration of the tissue. Panel d) shows the aggregate of blinded scoring of histopathology slides, where a score of 0 is completely normal tissue and 16 completely degraded. TNBS treated mice score highly whereas LES CM (Glutamax™)-treated mice have significant (1 way ANOVA n=10) protection against colitis (**** $P<0.0001$).

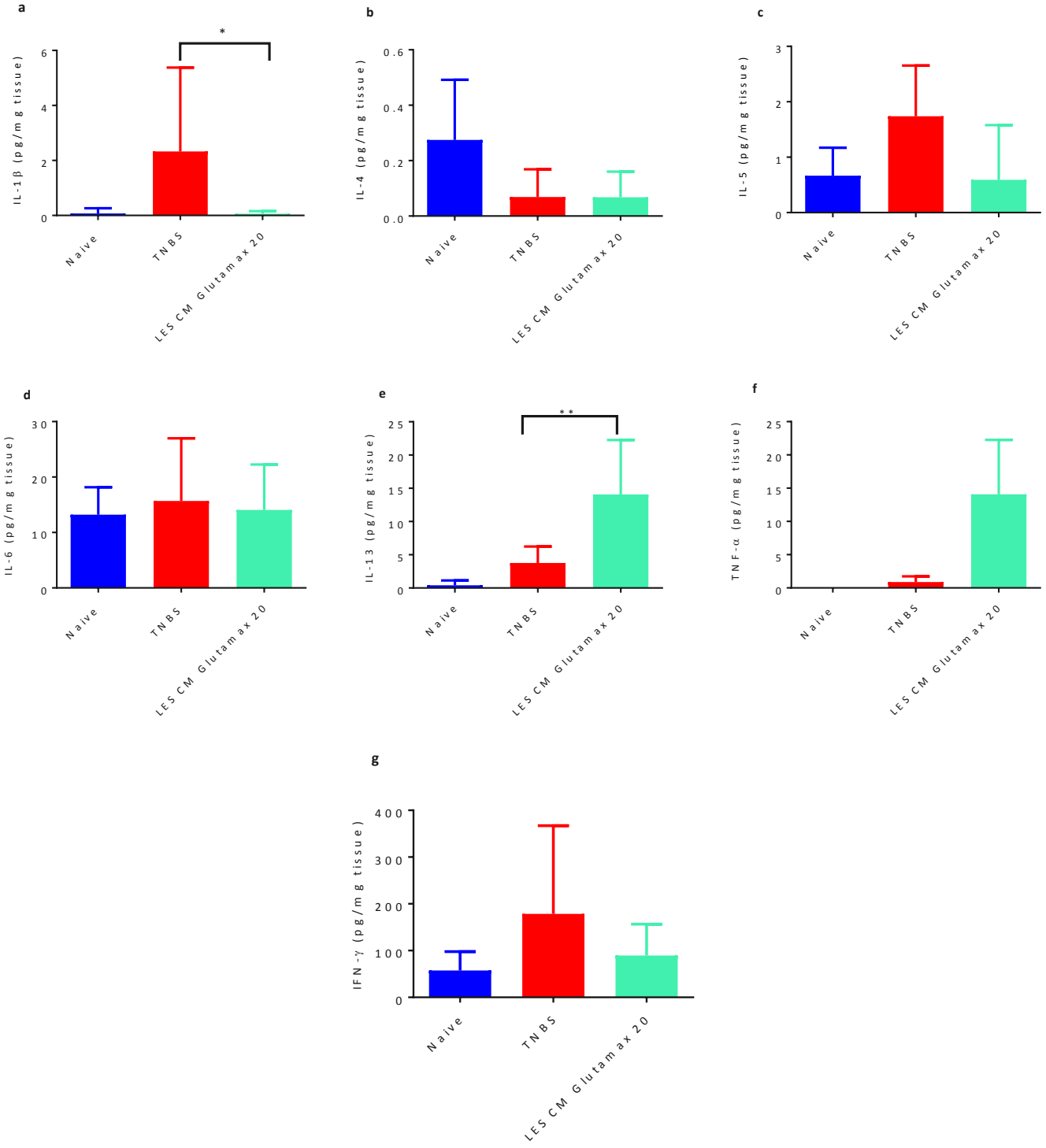


Figure 5.12. Changes in colon cytokine levels induced by treatment with 20 µg LES CM (Glutamax™) prior to induction of TNBS colitis in mice. Of the seven cytokines assayed here, two are significantly different from the TNBS control (1 way ANOVA). Cytokines were measured from colonic tissue cultured for 24 hours in complete media (a-g). LES (Glutamax)-treated mice had significantly lower levels of a) IL-1β (* P = 0.0145) and e) IL-13 (** P = 0.0024. There were no significant differences in the levels of IL-4 (b), IL-5 (c), IL-6 (d), TNF (f), and IFN-γ (g). All results are the aggregation of at least two independent experiments. Treatment groups received 20 µg material per mouse via i.p. injection (n=10).

5.5 Thin layer chromatography (TLC) separation of LES CM (Glutamax™) reveals four components that are protective in the TNBS colitis model.

After establishing that the CM fraction of LES (Glutamax™) can protect against TNBS colitis, further separation of components was achieved by TLC. Using a an Analtech-Uniplate silica gel plate with a chloroform: methanol (70:30) solvent system, 32.89 mg of LES CM (Glutamax™) extract was loaded onto the plate, separated and extracted as per the Materials and Methods; Chapter 3. The plate was visualised under both 254 and 365 nm and six fluorescent/absorbent bands were identified. In addition to this, five interceding non-absorbing bands were extracted. A representation of the extracted bands can be found in Figure 5.13 along with the extraction solvent and yield.

Samples were dried and weighed for yield then resuspended for analysis. On preparation, band 4 displayed differential solubility and was subsequently divided into two fractions, referred to here as Band 4 (precipitate) and Band 4s (supernatant fraction). Each band was administered to mice (20 μg per mouse via i.p. injection) prior to administration of TNBS for bioactivity assessment as previously described. Bands E_1 and E_2 were outside the sample separation area on the silica gel slide (area below the origin and above the solvent front) but were combined for TNBS testing as Band E. Figure 5.14 shows the weight loss curves of mice treated with eluate from bands A-E (a) and 1-6 (b).

TLC silica plate (Band)	Extraction Solvent	Yield (mg)
E_1	DCM:Methanol (70:30)	**1.4***
6	DCM:Methanol (70:30)	**2.31**
D	DCM:Methanol (70:30)	**0.98**
5	DCM:Methanol (70:30)	**6.64**
C	DCM:Methanol (70:30)	**1.45**
4	DCM:Methanol (70:30)	**14.86**
B	DCM:Methanol (70:30)	**1.83**
3	DCM:Methanol (70:30)	**1.35**
A	DCM:Methanol (60:40)	**1.44**
2	DCM:Methanol (60:40)	**2.12**
1	DCM:Methanol (60:40)	**0.44**
E_2	DCM:Methanol (70:30)	**1.4***

Figure 5.13. The representation of the large TLC silica gel separation plate of LES CM (Glutamax™) along with extraction and yield data. Bands A-E (green shading) displayed no fluorescence or absorbance at 254/365 nm. Bands 1-6 (grey shading) showed both fluorescence and absorbance at 254/365 nm. Bands were extracted in the solvent combinations, dichloromethane (DCM): methanol. The yield from each band is indicated; E_1 and E_2 were combined for TNBS testing and are indicated by *.

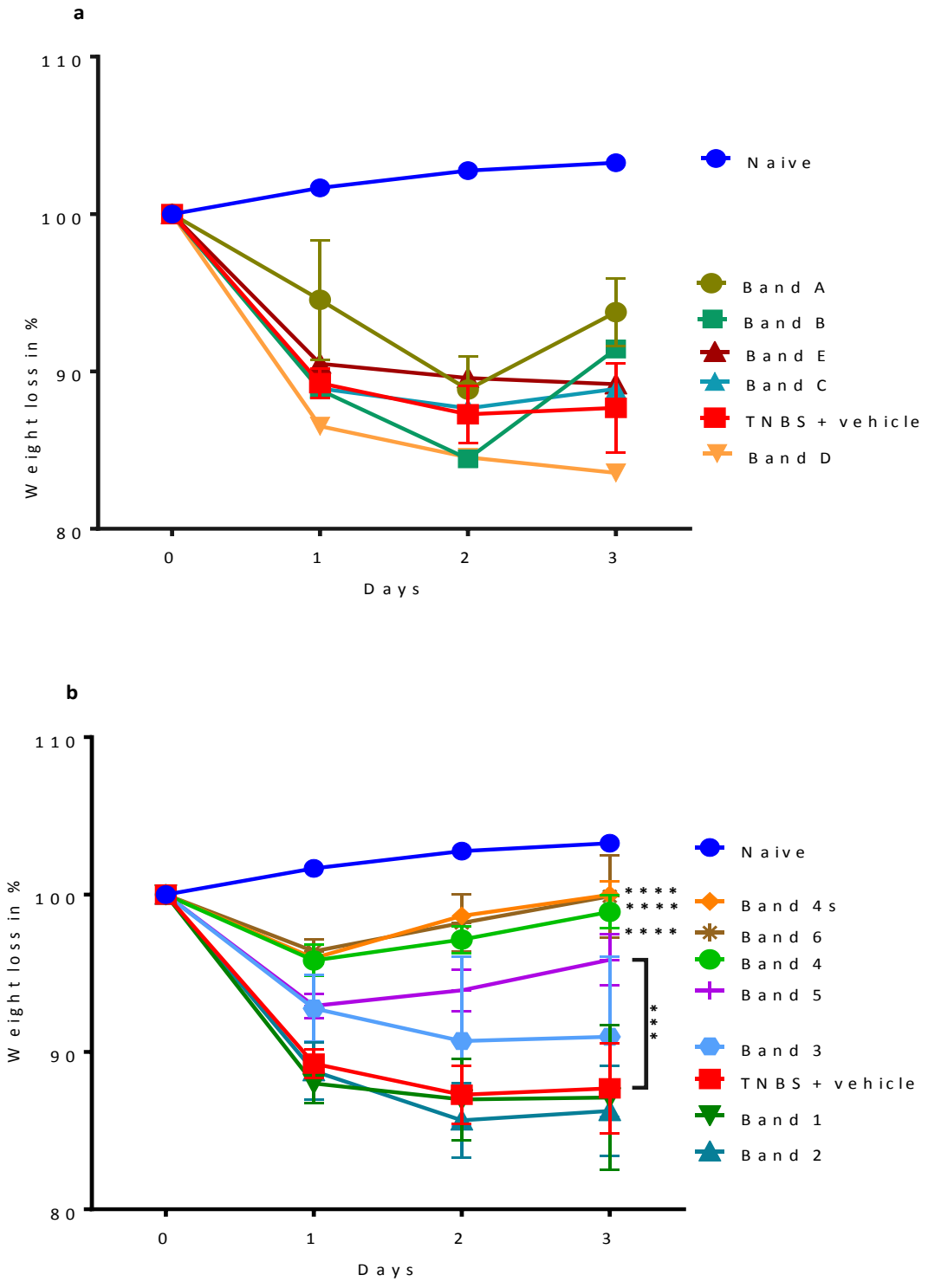


Figure 5.14. Four bands extracted from LES CM (Glutamax™) via TLC demonstrate protection against TNBS colitis weight loss. Here we tested 12 bands extracted from the TLC of LES CM (Glutamax™). Mice were treated with 20 µg of material via i.p. injection and four bands displayed protection against TNBS-induced weight loss. Band 4, 4s and 6 all significantly protect against weight loss (****P <0.0001), Band 5 (*** P = 0.0004) shows protection against chemical colitis when compared to the TNBS control. All other bands

showed no significant protection (bands A-E and bands 1-3). Band E (a) was combined from silica below the origin and above the reagent front of this separation and acts as a control of the silica gel TLC components that may be present. All differences were assessed by 1-way ANOVA when compared to the TNBS control. All results are the aggregation of at least two independent experiments. Treatment groups received 20 μg of material per mouse via i.p. injection (panel a; n=6, panel b; n=12).

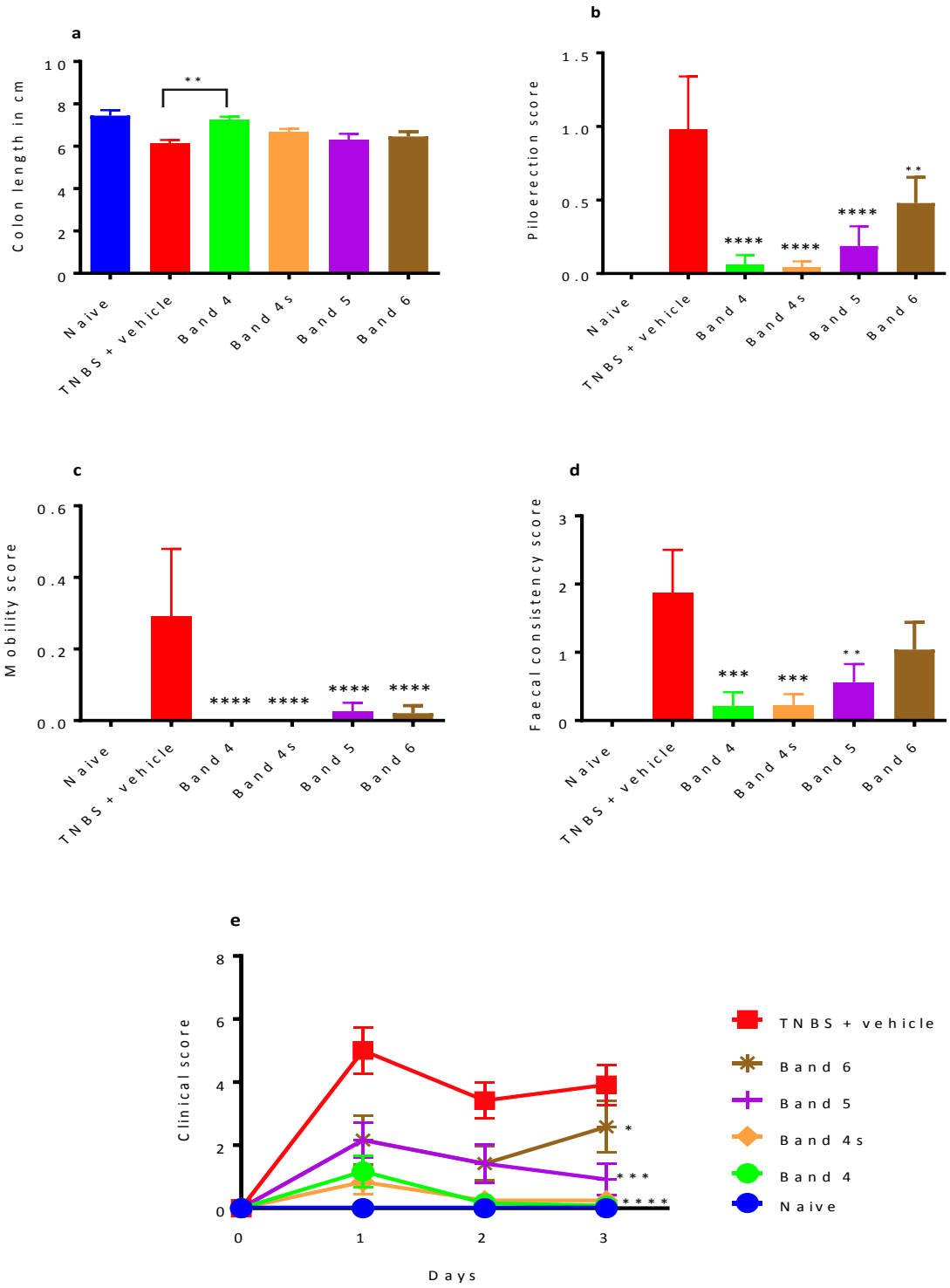


Figure 5.15. Mice treated with 20µg band extracts 4, 4s, 5 and 6 from LES CM (Glutamax™) have significantly lower clinical disease scores than the TNBS only treated controls. Piloerection score for mice treated with Band 4 from LES CM (Glutamax™) is significantly lower than the TNBS control group (a; ** P = 0.0022). The mobility score was significantly lower for bands from LES CM (Glutamax™) treatment group when compared to the TNBS control (b; bands 4, 4s and 5 (**** P = 0.0001) and band 6 (** P = 0.0018)). Similarly, stool consistency showed a marked improvement in bands from LES CM (Glutamax™)-treated mice when compared to TNBS controls (c; bands 4 and 4s

(*** P = 0.0002) and band 5 (** P = 0.0066). Finally, there was a significant difference in clinical scores of all bands from LES CM (Glutamax™)-treated mice compared to TNBS controls (d; band 4 and 4s (**** P<0.0001), band 5 (*** P = 0.008) and band 6 (* P = 0.017)). All differences were assessed by 1-way ANOVA when compared to the TNBS control. All results are the aggregation of at least two independent experiments. Treatment groups received 20 μg material per mouse via i.p. injection (n=12)

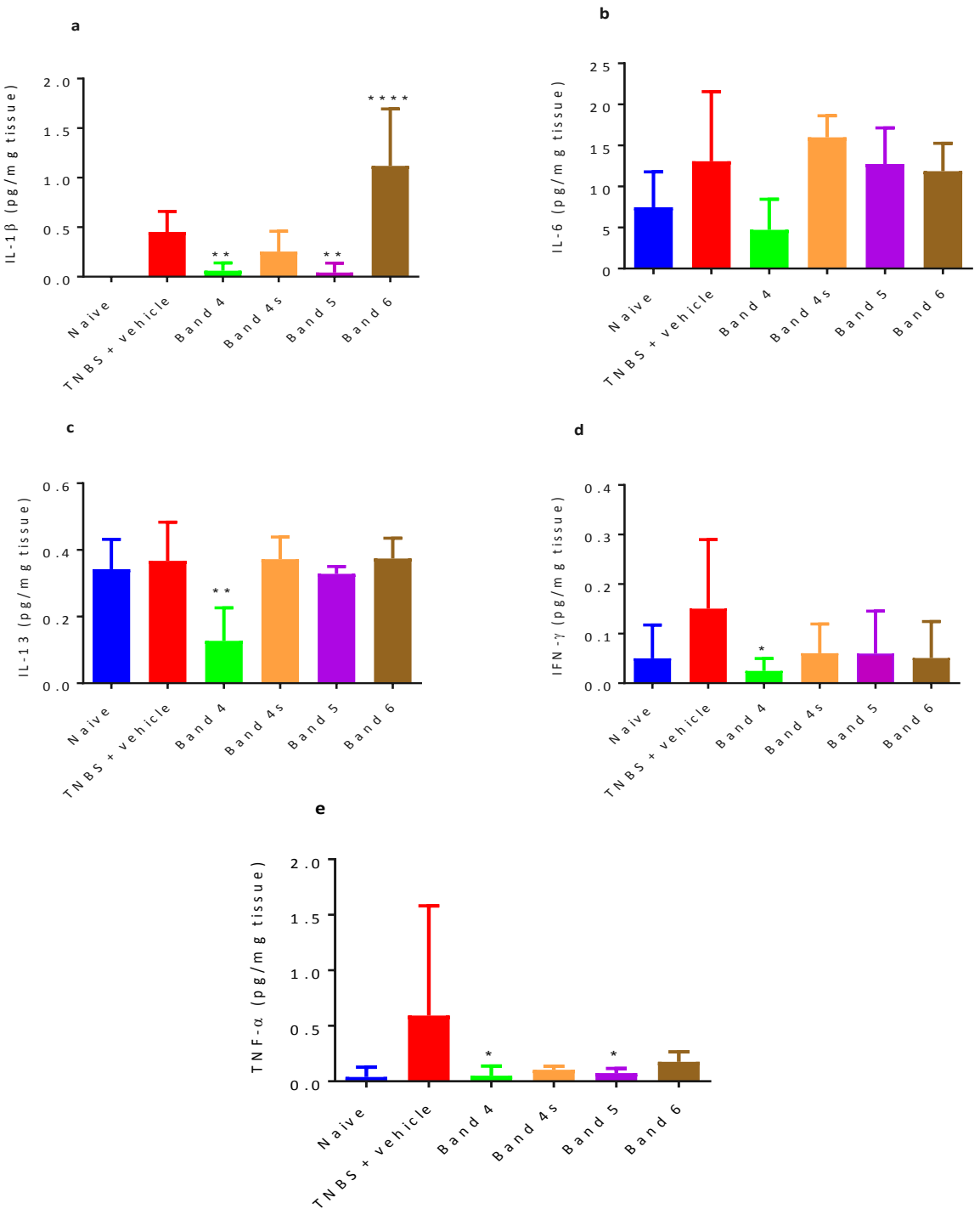


Figure 5.16. Changes in colon cytokine levels induced by treatment with 20 µg band extracts 4, 4s, 5 and 6 prior to the induction of TNBS colitis in mice. Of the five cytokines assayed here, four have at least one band that is significantly different from the TNBS control (1 way ANOVA). Cytokines were measured from colonic tissue cultured for 24 hours in complete media (a-e). Bands 4, 5 isolated from LES CM (Glutamax™)-treated mice had significantly lower levels of a) IL-1β; band 4 (** P = 0.0037) and band 5 (** P = 0.0016)

whereas levels of IL-1β for band 6 were significantly increased (**** P<0.0001). There were no significant changes in levels of b) IL-6 for any of the bands tested here. Only band 4 showed a significant reduction in c) IL-13 levels (** P = 0.0077) and d) IFN-γ levels (* P = 0.0209). Bands 4, 5 isolated from LES CM (Glutamax™)-treated mice had significantly lower levels of e) TNF; band 4 (*P = 0.0292) and band 5 (*P = 0.0411). All results are the aggregation of at least two independent experiments. Treatment groups received 20 μg material per mouse via i.p. injection (n=10).

Since protection was induced by only four bands (bands 4, 4s, 5 and 6), these bands were examined in more detail. Bands 4/4s, 5 and 6 are likely separate chemical entities as they are separated by interceding bands (bands C and D) which displayed no protection in the TNBS colitis bioactivity screen. These four bands have the highest therapeutic potential and their clinical indices were examined further (Figure 5.15).

Overall the most consistent protection was displayed by band 4 followed by bands 4s, 5 and finally band 6. While band 6 looked promising based on weight loss data, the clinical indices do not reliably reflect this. From these initial screening results, bands 4/4s display the greatest protection against TNBS colitis. It is likely that each of bands 4 and 4s have some constituent overlap and likely both contain the same active compound. The nature of protection of bands 5 and 6 may be distinct. To further understand the nature of protection, a limited palette of cytokines was assessed from colon cultures of these treatment groups - IL-1β, IL-6, IL-13, IFN-γ, and TNF (Figure 5.16). These cytokines are associated with IBD and inflammasome activation, and span the Th1, Th2, and Th17 arms of T helper cell responses. Each band generated a distinct cytokine profile. Band 6 showed significant protection against weight loss and the clinical symptoms of TNBS induced colitis, yet of the cytokines tested only IL-1β was significantly altered, and expression of this pro-inflammatory cytokine was, surprisingly, elevated in treated mice, in contrast to the clinical observations. Treatment with 20 μg of band 5 resulted in a suppression of IL-1β and TNF in mice after administration of TNBS. Despite significant protection in the clinical indices provoked by treatment with band 4s, there were no significant changes in any of the five cytokines tested. Treatment with 20 μg of band 4 displayed suppression of the pro-inflammatory cytokines IL-1β, IL-13, IFN-γ, and TNF.

5.6 Results Discussion

In summary, this chapter demonstrated that the TNBS chemical colitis model is a useful bioactivity screen for small molecules. The small molecule techniques routinely adopted in phytochemical research are suitable for the separation of hookworm LES components. I have also demonstrated that there are a number of small molecule fractions that elicit protection against TNBS colitis, possibly via different mechanisms of action (as reflected by distinct cytokine profiles). Four of these bands were isolated using the Folch extraction, which isolates molecules in the therapeutic window, indicating that there are a number of candidates that may be suitable to pursue for the treatment of inflammatory disease.

One of the major histological findings in LES Glutamax™- and LES CM Glutamax™- treated mice was the formation of lymphoid follicles. Lymphoid infiltration of tissues has been noted as a feature of *A. caninum* zoonosis [168]. Interestingly, *A. caninum* secretes the protein Neutrophil Inhibitory Factor (NIF) that blocks adhesion of these lymphocytes [162]. I hypothesise that NIF may be an evolutionary consequence of small molecule activation and aggregation of lymphocytes initiated by hookworm secreted small molecules. This may be one example of a complex and multilayered strategy that hookworms have evolved to evade their host.

One of the key cytokines associated with inflammasome production is IL-1β. The inflammasome are receptors and sensors involved in the regulation and the activation of inflammation of the innate immune system [401]. Here we show that IL-1β is consistently suppressed by all protective fractions tested as demonstrated in figures 5.7, 5.12 and 5.16. This indicates that the LES and its fractions may interact with the innate immune system to suppress inflammation. The mechanism by which this occurs has not been determined in this body of work.

Interestingly, the LES Glutamax fraction was able to elicit a significant difference in IL-17A cytokine levels when compared to the TNBS control (Figure 5.7). The cytokine IL-17A is known to play a role with both anti-inflammatory/regulatory responses. The ability to detect IL-17A it is perhaps unsurprising given that the parasite secretes a plethora of molecules into the surrounding tissues that have the potential to induce both pro- and anti-inflammatory responses. Inflammatory processes induced by the hookworm's aggressive

feeding behaviour may be partially mopped up by active secretion of anti-inflammatory molecules. Recent studies have also emphasised the importance of IL-17F as a target for the treatment of colitis [402] and future studies should include this avenue.

It is apparent from initial screens that culture conditions need to be validated when studying small molecules, as previous established methods may not be suitable. There are few established screening methods for anti-inflammatory activity for small molecules, and I have shown here that the TNBS model is suited to simultaneous profiling of a cross-section of immune responses. It is recognised that small molecules can be powerful inhibitors of the inflammasome [403, 404], yet I have been able to show here that small molecules may be involved in preventing other inflammatory processes. Indeed, two distinct bands of LES CM Glutamax™ isolated here are able to supress TNF production. This cytokine is a keystone target for many modern anti-inflammatory therapeutics and it is encouraging to highlight two possible small molecule candidates here.

5.7 The next chapter

The next chapter will present the characterisation of the LES of *A. caninum* using small molecule techniques with emphasis on those fractions that confer protection in TNBS-induced chemical colitis.

Chapter 6

The characterisation of small molecules found in the Excretory/Secretory component of adult *A. caninum*

Preamble

In this chapter I detail the molecular characterisation of *A. caninum* LES. This is the first description of the small molecule secretome of any helminth to date.

I have assembled gas chromatography/mass spectroscopy (GCMS) data from both derivatized and underivatized methods.

I describe these compounds with regards to known (reported in the literature) immunomodulatory activity and also in relation to the KEGG pathway maps of the human hookworm, *N. americanus*.

In addition, I compare the dataset assembled above with one assembled from LES cultured on a glucose substrate. This material (LES Glucose) was tested in the TNBS colitis model (Figure 5.1, Chapter 5) and mice had significantly poorer outcomes than the TNBS negative control (** P=<0.01). From the comparison of protective and a non-protective datasets it may be possible to highlight features that are responsible for protection in the TNBS colitis model.

Outputs from this chapter

The small molecule secretome of *Ancylostoma caninum*.
Shepherd C, Wangchuk P., Constantinoiu C., Navarro S., Wilson D., Kouremenos, K. A., De Souza, D. P.; Daly N., McConville, M. and A. Loukas
First Author

IN DRAFT; Target journal: International Journal for Parasitology

*This section was moved from Chapter 2 for brevity and to provide relevant background to the following chapter – the section has been framed by (…..) *

6.1 *A. caninum* biochemistry – our current state of play

(The dog hookworm has well described physiology and biochemistry which situates it as a translational model

The dog hookworm, *A. caninum*, was first identified in 1859 (Ercolani), and its physiology, morphology, biology and epidemiology have been well described, making it a good candidate as a model for translational research. Initial studies focussed on the description, harvesting, and optimisation of culture conditions for physiology experiments. It was determined that optimal conditions for harvested adult hookworms was a Krebs-Ringer solution with the addition of 50% dog serum, allowing adult worms to survive for up to 10 weeks [61]. These hookworms were able to reproduce and produce viable eggs. The addition of whole red blood cells did not have any effect on feeding activity and survival [61], and it was shown by other investigators that the majority of red blood cells pass through hookworms intact [405]. It was from these observations that it was assumed that hookworms get the majority of their nutrition from plasma rather than erythrocytes.

It is recognised that parasites convert their metabolic processes as they transition from free-living to parasitic lifestyles. However, little is known about the host cues and parasite molecular pathways that govern this process. Metabolic labelling studies in dog hookworm have determined that C^{14}-labelled glucose is not converted to glycogen [32], but is diverted into amino acid production [406] in a metabolism dominated by fermentative processes [407, 408]. Exposure of hookworm L3 to dog sera increases feeding rates [409], and large diffusible solutes (protein fraction) stimulate the consumption of glucose [61]. Cyanide inhibition studies show that the adult dog hookworm is capable of aerobic metabolism and has a tricarboxylic acid (TCA) cycle that is able to oxidize both pyruvic and succinic acids [410]. In addition, NADH respiration is not strongly coupled to oxidative phosphorylation, with evidence that hookworms lack respiratory control [411]. Adult *A. caninum* succinoxidase activity does not seem to be tightly coupled to the synthesis of ATP, and

external NADH oxidation that is not coupled to phosphorylation can occur. The low ratios of phosphorylation/oxidation may reflect loosely coupled pathways of respiration or the existence of two pathways of respiration - one coupled to the esterification of inorganic phosphate and another to the NADH pathway [411].

Fatty acids produced by *A. caninum* are major end products of metabolism. Labelling studies have shown that glucose is metabolised to produce acetate, propionate, and CO_2 while the amino acids L-valine and L-leucine are the precursors of isobutyric acid and isovaleric acid, respectively [88]. *A. caninum* adult worms consume larger quantities of oxygen than other intestinal nematodes [90], however, they have been shown to fix CO_2 to produce propionate from amino acids via a CO_2 fixation pathway [407, 408]. Adult hookworms display a slowing or cessation of metabolic fermentation in the presence of oxygen [411], and the presence of oxygen inhibits anaerobic glycolysis. The interactions of the end products of hookworm metabolism within the host have never been explored. Similarly, there has been no investigation of the role of secondary metabolites produced by hookworms and their roles in the host-parasite relationship.)

To date, very little is known about the small molecules that the hookworm produces in the context of the host-parasite interactions. The following results describe the data in terms of known biochemical information on *A. caninum* as well as the immunomodulatory activity and the anti-colitic properties of LES fractions described in the previous chapter.

6.2 Results

Pk #	Retention time (min)	# of carb-on	Kovat's Index ‡	Area %	MW	NIST Name†	ID match	Lit Ref Bioactivity‡	KEGG compound number
2	5.04	4	-	0.824	122	1,2,3,4-Butanetetrol, [S-(R*,R*)]-	83	-	-
3	5.17	9	-	0.592	186	Diethyl ethylidenemalonate	72	-	-
4	5.18	9	-	1.072	186	Itaconic acid diethyl ester	72	Immuno-modulatory, Anti-bacterial	Itaconic acid (C00490)
5	5.18	9	-	0.147	186	Diethyl ethylidenemalonate	73	-	-
6	5.21	4	-	0.125	122	Erythritol	77	-	C00503
7	5.23	8	-	0.187	136	Benzeneacetic acid	94	-	C07086
8	5.25	-	-	0.025	-	2(5H)-Thiopenone, 5-methyl-	-	-	-

9	5.26	5	844	0.005	114	2-Methyl-3-furanthiol	76	Unknown	-
10	5.26	8	-	0.217	136	Benzeneacetic acid	94	-	-
13	5.38	9	1253	0.094	165	Ethyl-2-pyridulacetate	78	Unknown	-
14	5.38	-	-	0.901	-	p-Methylbenzeneboronic acid	-	Anti-spasmodic	-
15	5.39	-	-	1.687	-	Pentaborane	72	-	-
16	5.46	6	1632	0.092	176	Vitamin C	70	Anti-oxidant	C00072
18	5.63	5	-	0.619	115	Methyl cyclopropylcarbamate	73	-	-
19	5.64	7	-	5.426	146	Butanoic acid, 2-(hydroxymethyl)-ethyl ester	70	-	-
20	5.66	13	1753	2.564	229	3-(2-Ethylhexoxy)propan-1-amine, N-acetate	64	Unknown	-
21	5.68	-	-	3.112	-	2H-Thiopyran-tetrahydro-4-methyl	70	-	-
22	5.72	0	-	0.283	66	Pentaborane (11)	68	-	-
23	5.86	8	-	0.035	157	Piperidine, TMS derivative	70	-	-
24	5.87	6	-	0.048	142	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl	64	-	-
25	5.89	3	-	10.55	92	Glycerol	84	-	C00116
26	5.99	13	-	0.022	230	Methoxyacetic acid, 6-ethyl-3-octyl ester	75	-	-
27	5.99	19	-	0.045	268	5 methyl-Octadecane	75	-	-
29	6.12	35	-	0.005	570	Pentratriacontane, 1-bromo-	76	-	-
30	6.13	12	-	0.043	200	Dodecanoic acid	86	-	C02679
31	6.17	14	-	0.035	206	2,4-Di-tert-butyphenol	84	-	-
32	6.22	15	-	0.032	242	Dodecanoic acid, 1-methylethyl ester	83	-	-
33	6.27	-	-	0.075	-	unknown	-	-	-
34	6.27	17	-	0.008	240	Hexadecane, 2-methyl	80	-	-
35	6.27	17	-	0.040	240	Heptadecane	73	-	C01816
36	6.37	10	-	0.197	161	2-Benzyl-1,3-oxazol-2-ine	88	Unknown	-
37	6.39	16	-	0.226	286	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	90	-	-
38	6.48	-	-	0.015	-	unknown	-	-	-
39	6.61	13	-	0.015	214	n-Heptyl hexanoate	75	-	-
40	6.81	10	-	0.107	172	Butyl 3-methylpentanoate	77	-	-
41	6.81	5	-	0.057	152	L-Aribinitol	70	-	-
42	6.88	8	-	1.885	135	Benzeneacetamide	92	-	-
44	6.96	11	-	0.431	218	4-Thia-2,6-diazabicyclo[3.2.0]hept-2-en-7-one	80	-	-
46	7.19	8	-	4.341	135	2-Acetyl-4-methyl pyridine	69	-	-
47	7.20	4	1229	13.34	122	Erythriol	76	Unknown	-
48	7.22	13	-	0.477	247	2-Benzylidene-5-oxooxazolidine-3-carboxylic acid, ethyl ester	85	-	-
49	7.23	12	-	8.584	221	Isoquinoline-4-carboxylic acid, 3-oxo-2,3,5,6,7,8-hexahydro-, ethyl ester	77	-	-
50	7.34	-	-	0.010	-	unknown	-	-	-
51	7.49	20	2000	0.048	282	Eicosane	87	Volatile oil	-
52	7.49	15	-	0.027	212	Tetradecane, 4-methyl-	81	-	-
53	7.49	19	-	0.023	268	5,5 Diethylpentadecane	84	Volatile oil	-

54	7.58	9	-	0.010	178	Phenacemide	73	Sodium channel blocker	C07428
55	7.59	14	-	0.018	212	Benzeneacetic acid, phenyl ester	85	-	-
56	7.66	12	-	0.007	222	Ethyl (phenylacetoxy)acetate	78	-	-
57	7.66	12	-	0.003	205	Morpholine, 4-(phenylacetyl)	61	-	-
58	7.6	8	-	0.010	150	Benzeneacetic acid, hydrazide	72	-	-
59	7.70	13	-	0.010	184	Dodecane, 2-methyl-	72	-	-
60	7.71	16	-	0.010	226	4-ethyl-Tetradecane	82	-	-
61	7.78	12	-	0.089	191	Diethyltoluamide	89	DEET	C10935
62	7.84	13	-	0.015	212	Octyl angelate	68	-	-
63	7.84		-	0.018	-	2-Butenoic acid, 2-methyl-, hexyl ester, (Z)-	-	-	-
64	7.85	15	-	0.025	212	Dodecane 2,6,10-trimethyl	62	-	-
65	7.95	4	-	0.020	114	2,4(1H,3H)-Pyrimidinedione, dihydro-	81	Unknown	-
66	8	10	1371	0.000	161	2-Benzyl 1,3-oxazol-2-ine	84	Unknown	-
67	8.01	-	-	0.015	-	Hexadecane	-	-	-
68	8.01	20	2000	0.022	282	Eicosane	87	Volatile oil	-
69	8.02	7	-	0.022	129	N-Ethyl-4-hydroxypiperidine	72	-	-
70	8.02	15	-	0.033	212	Tetradecane, 4-methyl	78	-	-
71	8.05	-	-	0.007	-	unknown	-		-
72	8.3	6	1335	0.002	123	Niacin	84	Immuno-modulatory	C00253
73	8.34	-	-	0.023	-	Oxazolidin-4-one, 5-benzylideno-2-thione-	-	-	-
74	8.35	8	-	0.022	118	Benzofuran	80	-	C14512
75	8.39	9	-	0.054	163	(2-Nitroallyl)-benzene	75	-	-
76	8.40	6	1632	0.070	176	Vitamin C	73	Anti-oxidant	C00072
77	8.40	12	-	0.030	191	Phenylacetimide, N-butyl	80	-	-
78	8.41	5	989	0.000	117	L-Valine	67	Immuno-modulatory	C00183
80	8.44	-	-	0.045	-	unknown	-	-	-
81	8.5	5	962	0.002	117	3-Hydroxy-N,N-dimethylpropanamide	72	Unknown	-
82	8.52	12	1585	0.152	185	1-(Phenylacetyl)pyrrole	92	Substrate/ column confounder	-
83	8.55	20	2000	0.002	282	Eicosane	85	Volatile oil	-
84	8.57	2	543	0.005	46	Formic Acid	91	Immuno-modulatory	C00058
85	8.62	5	787	0.002	118	Lactic acid ethyl ester	82	Immuno-modulatory	-
86	8.80	-	-	0.002	-	L-alanine ethylamide	-	Unknown	-
87	8.81	12	-	0.008	189	Benzenacetamide, N-methallyl-	66	-	-
88	8.91	4	987	0.045	114	2,4-Imidazolidinedione, 5-methyl-	93	Unknown	-
89	8.98	-	-	0.017	-	Isobutyl tricontyl ether	-	-	-
90	8.99	22	-	0.020	332	Benzeneacetic acid, 2 tetradecyl ester	78	-	-
91	9.23	8	-	0.114	118	Benzofuran	82	-	-
92	9.42	8	-	0.065	152	Benzeneacetic acid, 4-hydroxy-	84	-	-
93	9.53	7	992	0.008	145	L-Valine, ethyl ester	86	Unknown	-

94	9.57	-	-	0.047	-	unknown	-	-	-
95	9.63	12	1393	0.818	189	N-methallyl-Benzeneacetamide	80	-	-
96	9.68	11	-	0.013	178	Benzeneacetic acid 1-methylethyl ester	70	-	-
97	9.79	10	-	0.015	172	Pyrimidine, 5-hydroxy-4-phenyl-	66	-	-
98	9.81	10	-	0.308	158	1-(2-Aminophenyl)pyrrole	85	Unknown	-
99	9.95	-	-	0.003	-	unknown	-	-	-
100	10.03	16	-	0.005	256	n-Hexadecanoic acid	78	Substrate/ column confounder	C00249
101	10.13	-	-	0.008	-	unknown	-	-	-
102	10.26	4	1046	0.023	114	2,4(1H,3H)-Pyrimidinedione, dihydro-	92	Unknown	-
103	10.27	10	-	0.033	158	1-(2-Aminophenyl) pyrrole	83	Unknown	-
104	10.38	21	2006	0.002	296	5,5 Diethylheptadecane	79	Volatile oil	-
105	10.39	22	-	0.022	310	Eicosane 2,4-dimethyl-	72	-	-
106	10.39	19	-	0.003	268	5,5-Diethylpentadecane	66	Volatile oil	-
107	10.40	54	-	0.017	758	Tetrapentacontane	82	-	-
108	10.50	10	-	0.704	160	1H-Imadazole, 4,5-dihydro-2-(phenylmethyl)-	84	-	-
109	10.57	8	-	0.130	149	Benzene, (isothiocyanatomethyl)-	84	-	-
110	10.67	9	1253	0.013	152	2-(phenyl methoxy)-Ethanol	81	Unknown	-
111	10.78	-	-	0.012	-	unknown	-	-	-
113	10.79	20	2000	0.012	282	Eicosane	76	Volatile oil	-
114	10.80	14	-	0.737	212	9-Acridinamine, 1,2,3,4-tetrahydro-2-methyl-	-	-	-
115	10.82	14	-	0.729	212	1,2,3,4-tetrahydro-2-methyl-9-Acridinamine	70	Substrate/ column confounder	-
117	10.93	-	-	0.008	-	unknown	-	-	-
118	11.11	16	1942	0.246	256	n-Hexadecanoic acid	87	Substrate/ column confounder	-
119	11.12	8	-	0.022	149	Benzene, (isothiocyanatomethyl)-	72	-	-
120	11.15	14	-	0.043	228	Benzene ethanethioic acid, S-phenyl ester	74	-	-
121	11.16	15	-	0.144	229	N-ethyl-N-(4-methylpenta-2,4-dienoyl)benzylamine	77	-	-
122	11.21	9	-	0.010	221	1-Alanine, N-(5-chlorovaleryl)-,methyl ester	68	Unknown	-
123	11.29	11	-	0.023	226	Phenobarbital	84	-	-
124	11.42	20	-	0.015	282	3-Ethyl-3-methylheptadecane	82	-	-
125	11.42	15	-	0.017	212	5-methyl tetradecane	82	-	-
126	11.43	29	-	0.015	408	Nonacosane	72	-	C08384
127	11.60	9	-	0.012	326	2-Propanol, 1-chloro, phosphate(3:1)	74	-	-
128	11.69	15	-	0.079	212	1(2H)-Pentalenone, hexahydro-4-(phenylmethylene)-	62	-	-
129	11.69	-	-	0.040	-	unknown	-	-	-
130	11.71	3	525	0.010	77	Methyl nitrate	81	Unknown	-
131	11.78	13	-	0.333	215	4-Benzyloxy-2-methylpyridine 1-oxide	84	-	-
132	11.79	15	-	0.219	210	2-Propanone, 1,3-diphenyl-	80	-	-

133	11.79	6	1489	0.048	160	N-L-alanyl-L-Alanine	78	Unknown	-
134	11.80	8	-	0.299	120	Phthalan	77	-	-
135	11.9	14	1923	0.002	246	Phenyl acetic acid, 2-chlorophenyl ester	80	Unknown	-
136	12.02	10	-	0.298	158	1(2-Aminophenyl) pyrrole	86	Unknown	-
137	12.07	-	-	0.085	-	unknown	-	-	-
138	12.08	15	-	0.687	210	2-Propanone, 1,3-diphenyl-	93	-	-
139	12.44		-	0.013	-	unknown	-	-	-
140	12.46	16	-	0.033	278	1,2 Benzenedicarboxylic acid, (2-methylpropyl) ester	91	-	-
141	12.47	16	-	0.018	278	1,2-Benzenedicarboxylic acid, bis(2-methypropyl) ester	86	-	-
142	12.56	15	1736	0.242	210	1,3-diphenyl-2-Propanone	83	Substrate/ column confounder	-
143	12.63	15	-	0.400	210	2-Propanone, 1,3-diphenyl-	81	-	-
144	12.70	-	-	0.010	-	unknown	-	-	-
145	12.75	15	-	0.697	210	1,3-diphenyl-2-Propanone	84	Substrate/ column confounder	-
146	12.90	11	-	0.030	207	Glycine, N-(phenylacetyl)-, methyl ester	81	Substrate/ column confounder	-
147	13.08	7	-	0.015	136	p-methylbenzeneboronic acid	73	Anti-spasmodic	-
148	13.08	8	-	0.015	135	Benzeneacetamide	95	-	-
150	13.11	8	953	0.072	142	Acetaldehyde, bis(1-methylethyl) hydrazone	84	Unknown	-
149	13.13	11	1694	1.839	207	Glycine, N-(phenylacetyl)-, methyl ester	92	Substrate/ column confounder	-
151	13.17	15	-	0.035	210	1,3-diphenyl-2-Propanone	83	Substrate/ column confounder	-
152	13.36	20	-	0.152	312	Dodecanoic acid, isoctyl ester	89	-	-
153	13.37	-	-	0.027	-	unknown	-	-	-
154	13.56	7	-	0.032	136	p-Methylbenzeneboronic acid	80	Anti-spasmodic	-
155	13.56	7	794	0.084	92	Toluene	87	Substrate/ column confounder	C01455
156	13.73	12	-	0.032	186	1H-Benzo[d]pyrrole[1,2-a]imidazole, 6,7-dimethyl-2,3-dihydro	73	-	-
157	13.77	16	-	0.022	247	Phenylacetamide, N,N-dibutyl-	68	-	-
158	14.04	-	-	0.007	-	unknown	-	-	-
159	14.09	-	2054	0.017	-	Benzylacetic acid decyl ester	70	-	-
160	14.29	-	-	0.008	-	unknown	-	-	-
161	14.41	9	-	0.149	212	Imidazolo[2,1-b]thiazol-2 carboxcylic acid 5,6-dihydro-3-methyl-, ethyl ester	63	-	-
162	14.55	-	-	0.007	-	unknown	-	-	-
163	14.56	8	1292	0.508	137	3-Pyridinepropanol	70	-	-

164	14.91	15	1736	0.080	210	1,3-diphenyl-2-propanone	81	Substrate/ column confounder	-
165	15.29	7	-	0.033	136	p-Methylbenzeneboronic acid	81	Anti-spasmodic	-
166	15.32	-	-	0.013	-	unknown	-	-	-
167	15.35	5	-	0.194	129	L-Glutamine	86	-	C00064
168	15.44	7	794	0.010	92	Toluene	82	Substrate/ column confounder	C01455
169	15.57	18	-	0.234	351	N-Benzyl-4-(2,4-dichloro-phenoxy)-N-methyl-butyramide	63	-	-
170	15.58	11	1358	0.182	205	Mandelonitrile, TMS derivative	66	Substrate/ column confounder	-
171	16.02	7	-	0.052	154	2,4(1H,3H)-Pyrimidinedione, 1,3,5-trimethyl-	77	-	-
172	16.22	-	-	0.018	-	Decane, 1-iodo-	-	-	-
173	16.23	19	1805	0.020	268	5,5-Diethylpentadecane	81	Volatile oil	-
174	16.25	-	-	0.010	-	unknown	-	-	-
175	16.40	20	-	0.020	334	1,2-Benzenedicarboxcylic acid, butyl octyl ester	87	-	-
176	16.41	-	-	0.012	-	unknown	-	-	-
177	16.60	13	-	0.094	204	Phenylacetic acid, 3-methylbut-2-enyl ester	77	-	-
178	16.79	5	1180	0.254	129	DL-Pyroglutamic acid	86	Substrate/ column confounder	C01879
179	16.82	10	-	0.010	163	Phenylacetamide, N-ethyl-	69	-	-
180	17	19	1805	0.002	268	5,5 Diethylpentadecane	76	Volatile oil	-
181	17.00	8	-	0.015	172	Thiourea, N-cyclohexyl-N'-methyl-	62	-	-
182	17.41	18	-	0.010	276	Benzylacetic acid decyl ester	71	-	-
183	17.82	18	2187	0.109	284	Octadecanoic acid	90	-	C01530
184	18.12	20	-	0.107	312	Acetic acid n-octadecyl ester	95	-	-
185	18.18	10	1858	0.005	210	3-Methyl-1,4-diazabicyclo[4,3,0]-dione N-acetyl	75	Unknown	-
186	18.38	20	2000	0.025	282	Eicosane	84	Volatile oil	-
187	18.39	44	-	0.022	618	Tetratetracontane	83	-	-
188	19.53	13	-	0.129	204	Phenylacetic acid, 3-methylbut-2-enyl ester	77	-	-
189	19.58	18	2054	0.010	276	Benzeneacetic acid, decyl ester	74	Unknown	-
190	20.89	7	1795	0.015	154	Pyrrole[1,2-a]pyrazine-1,4-dione, hexahydro-	83	Antimicrobial	-
191	21.31	14	-	0.025	208	7,11-Dimethyldodeca-2,6,10-trien-1-ol	67	-	-
192	21.63	8	1217	0.022	143	N-hexyl Acetimide	77	Unknown	-
193	21.98	19	1980	0.035	244	Triphenylmethane	90	Immuno-modulatory	-
194	22.23	-	-	0.018	-	2,6,10,14-tetramethyl-Octadecane	-	-	-
195	22.29	40	-	0.017	562	Octatriacontane, 3,5-dimethyl-	71	-	-
196	22.65	-	-	0.144	-	unknown	-	-	-
197	22.69	15	2185	0.010	284	Benzenesulfonic acid 4 methyl-octyl ester	65	Unknown	-
199	23.01	10	1781	0.038	194	2,5-cyclohexadiene-1,4-dione 2,5 methyl-3,6-bis(methylamino)	72	Unknown	-

200	23.79	16	-	0.067	276	1,2-Cyclohexanedicarboxylic acid, 4-phenyl-, dimethyl ester	66	-	-
201	23.81	-	-	0.062	-	unknown	-	-	-
202	24.27	-	-	0.022	-	unknown	-	-	-
203	24.3	10	1781	0.022	194	2,5-cyclohexadiene 1,4-dione 2,5 dimethyl-3,6-bis(methylamino)	72	Unknown	-
204	24.57	10	-	0.007	193	N-(Phenylacetyl)glycine	61	-	-
205	25.10	19	3309	0.054	412	1,6-Anhydro-4-[2-(pyrazolidinyl)ethylamino]-2-0-tosyl-4-deoxy-b-d-glucopyranose	80	Unknown	-
206	25.65	12	2141	0.048	263	D-Alanine, N-(5-chlorovaleryl)-butyl ester	78	Unknown	-
207	25.68	14	-	0.047	291	D-Alanine, N-(5-chlorovaleryl)-, hexyl ester	73	-	-
208	25.80	-	-	0.005	-	unknown	-		-
209	26.23	6	-	0.090	127	4-Methyleneproline	86	Substrate/ column confounder	-
210	26.26	14	2141	0.010	291	D-Alanine, N-(5-chlorovaleryl)-,hexyl ester	76	Substrate/ column confounder	-
211	26.49	15	-	0.084	343	N-(4-Methylpropyl-glutamyl)-glycine ethyl ester	76	-	-
212	26.54	20	-	0.007	282	Eicosane	80	Volatile oil	-
213	26.75	6	1229	0.087	127	4-Methyleneproline	81	Substrate/ column confounder	-
214	26.97	6	-	0.010	143	L-Proline, 5 oxomethyl ester	77	-	-
215	27.20	20	-	0.054	282	Eicosane	77	Volatile oil	-
216	27.21	24	-	0.050	338	Tetracosane	86	-	-
217	27.29	14	2065	0.015	288	Benzaldehyde 3-hydroxy-4-benzyl-oxy-	76	β_3 adrenergic receptor agonist	-
218	27.62	13	-	0.064	278	Piperidin-2-thione-6-one, 5(benzyloxycarbonyl)amino	65	-	-
219	27.86	14	1898	0.002	250	5,10-Diethyoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a1'2']pyrazine	76	Unknown	-
220	27.91	-	-	0.003	-	unknown	-	-	-
221	28.17	11	2005	0.003	168	9H-Pyrido[3,4-b]indole	86	Unknown	-
222	28.26	22	-	0.028	340	Eicosyl acetate	87	-	-
223	28.26	20	-	0.043	312	Acetic acid n-octadecyl ester	88	-	-
225	28.42	23	-	0.059	346	Benzeneacetic acid, 3-pentadecyl ester	79	-	-
224	28.44	19	1805	0.007	268	5,5 Diethylpentadecane	80	Volatile oil	-
226	29	13	2492	0.002	264	L-Glutamine, N2-(phenylacetyl)-	67	Unknown	-
227	29.76	8	-	0.037	165	Furo[3,4-c]pyridine-3,4(1H,5H)-dione, 6-methyl	73	-	-
228	29.77	-	-	0.059	-	unknown	-	-	-
229	29.83	16	-	0.254	226	Hexadecane	64	-	-
230	29.96	8	2097	0.059	217	l Alanyl-l-glutamine	79	Substrate/ column confounder	-
231	30.14	13	-	0.027	219	Acetamide, N-tetrahydrofurfuryl-2-phenyl-	-	-	-

232	30.16	11	-	0.054	236	Acetamide, 2-phenyl-N1-(2-oxo-4-mercapto-3-azetidinyl)	84	-	-
233	30.21	-	-	0.017	-	unknown	-	-	-
234	30.34	13	-	0.052	247	2-Benzylidine-5-oxooxazolidine-3-carboxcylic acid	65	-	-
235	30.62	9	1473	0.007	178	Phenacemide	-	Sodium channel blocker	-
236	30.98	9	1125	0.005	135	Amphetamine	73	Immuno-modulatory	-
237	31.38	17	-	0.008	334	Piperazine, 1-benzyl-4(4-fluorophenylsulfonyl)-	63	-	-
238	31.43	8	-	1.836	175	1H-Tetrazol-5-amine, 1(phenylmethyl)	72	Kinase inhibitor	-
239	31.59	-	-	0.065	-	unknown	-	-	-
240	31.59	20	-	0.059	282	Eicosane	82	Volatile oil	-
242	31.80	10	1847	0.227	192	Benzeneacetamide, N-[2-(hydroxyimino)ethyl]-	81	Unknown	-
241	31.98	-	-	0.032	-	1,3-Propanediol, 2-(phenylmethyl)-	-	-	-
243	32	8	-	0.002	175	1H-Tetrazol-5-amine, 1-(phenyl methyl)-	72	Kinase inhibitor	-
244	32.04	-	-	0.082	-	unknown	-	-	-
245	32.49	-	-	0.018	-	unknown	-	-	-
246	32.54	-	-	0.062	-	unknown	-	-	-
247	33.05	-	-	0.005	-	unknown	-	-	-
248	34.56	-	-	0.040	-	4H-1,2,4-triazolium, 3-mercapto-1,5-dimethyl-4-(1-methylet	-	-	-
249	34.58	14	-	0.028	227	4(4-Phenyl-1H-imidazol-zyl) piperidine	65	-	-
250	35.36	11	1552	0.005	172	N-[2-Cyanoethyl]styrenimine	63	Unknown	-
251	35.81	-	-	0.052	-	unknown	-	-	-
252	35.83	-	-	0.052	-	unknown	-	-	-
253	36.04	13	-	1.049	234	Benzyl ethyl maleate	62	-	-
254	36.19	10	1430	0.003	268	Decane, 1-iodo-	78	Unknown	-
255	36.69	-	-	0.032	-	unknown	-	-	-
256	36.71	13	1557	0.025	206	Pentyl phenylacetate	71	Unknown	-
257	37.11	-	-	0.114	-	unknown	-	-	-
258	37.13	23	-	0.296	346	Benzeneacetic acid, 4-pentadecyl ester	74	-	-
259	37.15	36	-	0.104	506	Hexatriacontane	82	-	-
260	37.73	11	1784	0.120	204	Imidazolidin-2-one, 1-phenylacetyl	73	Kinase inhibitor	-
261	37.75	13	-	0.042	219	Acetamide, N-tetrahydrofurfuryl-2-phenyl	71	-	-
262	37.76	-	-	0.045	-	unknown	-	-	-
263	38.12	19	2097	0.005	290	Dodecanoic acid, phenylmethyl ester	62	Anti-microbial	-
264	38.38	8	2097	0.411	217	l-Alanyl-l-glutamine	85	-	-
265	38.68	12	1841	0.030	218	2,5-Piperazinedione, 3 methyl-6-(phenylmethyl)	86	Unknown	-
266	38.83	22	-	0.013	363	L-Cysteine, S-(triphenylmethyl)-	73	-	-
267	39.16	13	1727	0.013	276	Isobutyl propane-1,3-diyl dicarbonate	73	Unknown	-

268	39.2	19	-	0.002	441	Folic acid	68	Immuno-modulatory	-
269	39.21	19	-	0.040	276	2-Benzyl-8-methyl-1H-piperidino[4,3-b]indole	68	-	-
270	39.3	12	1841	0.002	218	2,5 Piperazinedione, 3-methyl-6-(phenylmethyl)	86	Kinase inhibitor	-
271	39.4	9	2115	0.226	244	2-(5-Methyl-3,6-dioxopiperazin-2-yl methyl)-malonic acid	84	Unknown	-
272	39.58	11	1423	0.020	220	Pivalic, 6-chlorohexyl ester	65	Antifungal	-
273	39.80	-	-	0.008	-	unknown	-	-	-
274	40.13	-	-	0.018	-	unknown	-	-	-
275	40.16	11	-	0.032	176	1-phenyl-2,4-Pentanedione	73	-	-
276	40.45	7	-	0.008	203	Glycyl-l-glutamine	63	-	-
277	40.46	36	-	0.129	506	Hexatriacontane	93	-	-
278	40.46	20	-	0.130	282	Eicosane		Volatile oil	-
279	40.61	-	-	0.027	-	unknown	-	-	-
280	40.62	-	-	0.018	-	unknown	-	-	-
281	40.75	-	-	0.064	-	unknown	-	-	-
282	40.79	-	-	0.033	-	unknown	-	-	-
283	40.80	12	1953	0.007	263	D-Alanine, N-(5-chlorovaleryl)-,butyl ester	65	Unknown	-
284	40.80	13	-	0.338	220	Benzeneacetic acid, (tetrahydrofuranyl)methyl ester	82	Unknown	-
285	41.12	13	1689	0.107	220	Benzeneacetic acid, (tetrahydrofuranyl)methyl ester	81	Unknown	-
286	41.15	14	-	2.661	291	3-Diethylamino-1-(3-nitro-phenyl)-pyrrolidine-2,5-dione	70	-	-
287	41.33	19	-	0.032	288	Benzeneacetic acid, 10-undecenyl ester	71	-	-
288	41.39	25	-	0.027	398	Docosyl ethyl carbonate	67	-	-
289	41.4	24	-	0.346	390	Diisooctyl phthalate	94	-	-
290	41.41	11	1780	0.007	204	2,5 Piperazinedione, 3-(phenylmethyl)-	75	Unknown	-
291	41.70	15	-	0.042	233	Phenylacetamide, N-(hept-2-yl)-	67	-	-
292	41.97	25	-	0.025	372	9-Octadecanoic acid (Z)-, phenylmethyl ester	68	-	-
293	42.20	-	-	0.075	-	unknown	-	-	-
294	42.22	-	-	0.112	-	unknown	-	-	-
295	42.25	19	1805	0.007	268	5,5 Diethylpentadecane	78	Volatile oil	-
296	42.46	14	-	0.013	246	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	62	-	-
297	42.49	14	-	0.038	246	3-benzyl-6-isopropyl-2,5-Piperazinedione	62	-	-
298	42.56	12	1953	0.079	263	D-Alanine, N-(5-chlorovaleryl)-,butyl ester	71	Unknown	-
299	42.57	36	-	0.254	506	Hexatriacontane	90	-	-
300	42.73	13	-	0.085	219	Acetamide, N-tetrahydrofurfuryl-2-phenyl-	68	-	-
301	42.84	12	2162	0.258	236	dl-Alanyl-l-phenylalanine	65	Substrate/ column confounder	-
302	42.87	20	-	0.025	310	Cyclopentanecarboxcylic acid, tetradecyl ester	71	-	-
303	42.90	15	-	0.602	259	Isoindole-1,3-dione, perhydro-2-benzyloxy-	65	-	-

304	43.01	19	2120	0.022	296	Cyclopentanecarboxcylic acid, tridecyl ester	75	Unknown	-
305	43.23	18	2517	0.040	261	Benzeneacetamide, N-(2-napthyl)-	87	Unknown	-
306	43.23	-	-	0.092	-	unknown	-	-	-
307	43.27	20	-	0.032	338	cis-Cyclohex-4-en-1,2-dicarboxcylic acid, di(isohexyl)ester	68	-	-
308	43.30	-	-	0.117	-	unknown	-	-	-
309	43.50	-	-	0.013	-	unknown	-	-	-
310	43.83	-	-	0.010	-	unknown	-	-	-
312	43.91	7	1292	0.002	139	Rolziacetam	67	Unknown	-
313	44.02	11	-	0.099	154	2-Heptene, 5-ethyl-2,4-dimethyl-	68	-	-
314	44.09	-	-	0.005	-	unknown	-	-	-
315	44.20	30	-	0.070	422	Triacontane	87	-	C08389
316	44.21	15	-	0.040	219	Benzene methanamine, N-butyl-N-(2-methylpropyl)	70	-	-
317	44.21	20	-	0.112	282	Eicosane	91	Volatile oil	-
318	44.34	6	967	0.921	127	Pyridine, 2-chloro-6-methyl-	65	-	-

Table 6.1. The underivatized LES secretome of the helminth *A. caninum* as determined by GCMS and FID. Identity of chromatogram features from LES Glutamax™ determined from comparison with the NIST database. ID score match is an algorithm-based index of confidence in the identity of the small molecule from each peak. Molecules are colour coded on the basis of confidence of identity; xx indicates a very good match of 100-85; xx indicates a good match with an ID confidence of 84-70; xx indicates a fair match with an ID confidence of 69-60; those molecules with matches below 60 xx are considered tentative ID matches. Those molecules with poor confidence or of unknown origin in the NIST database are left uncoloured. Molecules that have low ID matches but occur consistently across multiple replicates (n=3) are indicated as xx and highlighted in the ID match column. All identified molecules were checked against the ChemSpider database for known bioactivity and reported Kovat's index recorded (molecules that had no bioactivity reported were also checked by a NCBI journal search - IUPAC name and "anti-inflammatory"). Finally, the KEGG compound number is also reported in this table.

†NIST name from http://webbook.nist.gov/chemistry/

‡As sourced from ChemSpider Bioactivity and NCBI PubMed database search (https://www.ncbi.nlm.nih.gov/pubmed/)

Polar Metabolite	Relative Absorbance	-/+ SD	Pathway*	KEGG† Compound #
Maltose	0	0		C00208
Tyrosine	161.3333	76.53569		C00082
Aspartic acid	193.3333	43.3687		C00049
Hexadecanoic acid	456.6667	291.4066		C00249
Histidine	460.3333	120.8709		C00135
Hypoxanthine	880.5	292.3539		C00262
Tryptophan	890.1667	602.4303		C00078
Glutamine	1050.333	307.2001		C00064
Glucosamine	1068.167	205.134		C00329
GABA	1187	559.3848	GABA	C00334
D-Galactose-6-phosphate	1198.667	541.3814		C01113
2-hydroxyisocaproic acid	1268.5	262.3196	BCA	C03467
meso-Erythritol	1800.833	333.2793	T1R3 receptor/ligand?	C00503
4-hydroxyphenylacetic acid	1999.667	199.1503		C00642
Lysine	2155	445.4069		C00047
Isocitric Acid	2327.5	265.5293		C00311
D-Xylose	2540	394.155		C00181
Asparagine	2614.167	620.3666		C00152
3-methyl crotonic acid	3431.833	785.0552	Branch chain amino acid Degeneration	C01771
Glycerol-2-phosphate	4206.167	574.3366	Glycolysis	C02979
beta-alanine	5966	983.6092		C00099
Methionine	6067.833	1084.094		C00073
Fructose	7195.5	912.4014		C00095
Isoleucine	8332	1238.951	Branch chain amino acid	C00407
Glucose	9335.5	4075.877	Glycolysis	C00031
Talose	9744.333	2788.72		C06467
Malic acid	13499.83	918.0958	TCA cycle	C00149
Threonine	15477.33	3822.949		C00188
Citric acid	17206.67	1448.048	TCA cycle	C00158
Phenylalanine	21077.17	6967.139		C00079
Glucose 6-phosphate	23385	2793.209	Glycolysis	C00092
Succinic acid	24257	1288.725	TCA, GABA	C00042
Fructose 6-phosphate	25961.67	5081.596	Glycolysis	C00085
Ribitol	30008.33	5675.8		C00474
D-Ribose	50717.83	8421.589		C00121
Glutamic acid	57999.33	18175.52	TCA cycle, GABA, amino acid synthesis	C00025
Alanine	98958.83	23616.5		C00041
Proline	150547.2	31383.81		C00148
Lactic Acid	203525.3	17522.41	EP	C00186
Urea	204689.8	10672.9		C00086
Adenine	331455.5	38317.57		C00147

Glycerol	1713390	102051.9	GlycoLipid	C00116
Glycolic acid	3133615	318185.7		C00160
Pyroglutamic acid	7929140	719923		C01879

Table 6.2. Polar metabolite components of LES Glutamax™ as determined by TMS derivatization and GCMS analysis (Bio21 Institute, Melbourne University). The metabolites presented in this table are molecules detected and ranked in order of abundance. The KEGG compound number is recorded for each molecule as well as the biochemical pathway that is commonly associated with that molecule. Abundance is the average of number of sample replicates (n=6).

*Biochemical pathways associated with immune function.

†KEGG compound number was sourced from http://www.genom.jp/kegg/compound

6.3 The small molecule secretome of adult *A. caninum*

In this section I present both the derivatized and the underivatized GCMS data for *A. caninum* LES harvested from culture in 1 % Glutamax™. Combined, these data provide a first sweep characterisation of the small molecule ES component produced by *A. caninum*. The data in Table 6.1 presents the underivatized GCMS data for LES (Glutamax™) and Table 6.2 presents the derivatized GCMS data for LES (Glutamax™). In Figure 6.1, I present this combined data mapped against the KEGG biochemical pathways of the human hookworm *N. americanus*.

Table 6.1 characterizes shows the underivatized data from LES Glutamax™ using a small molecule approach. It was possible to identify 317 features in this dataset with 15.7% being completely novel features (unassignable/unidentified). The fragment pattern of each peak on the gas chromatogram was compared to the National Institute of Standards and Technology (NIST) database (http://webbook.nist.gov/chemistry/). These matches were generated automatically using the Shimadzu 2010 data analysis program and then checked manually by comparing the 20 top fragment matches with the chromatogram peak fragmentation patterns. The chromatogram peaks were then checked in a blinded fashion, files were de-identified, and peaks were manually checked against the NIST database. Identities were then assigned on the basis of the highest algorithmic and fragmentation match. The retention time, the relative amount of each feature (%) and the reported molecular weight, number of carbons and Kovat's index (if reported) were recorded. The molecules were then ranked in order of confidence of identification as highlighted in Table 6.1. Molecules identified were then crosschecked in ChemSpider

(http://www.chemspider.com). In addition, a search for these molecules in NCBI PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) by their IUPAC (International Union of Pure and Applied Chemistry) name and the search term “anti-inflammatory” was recorded in the reported bioactivity column. Finally, molecules were searched in the KEGG (Kyoto Encyclopaedia of Genes and Genomes) database (http://www.genom.jp/kegg/) and their assigned compound numbers recorded.

The four most abundant features in this database are erythriol (13.34%), glycerol (10.55%), isoquinolone-4-carboxcylic acid, 3-oxo-2, 3, 5, 6, 7, 8-hexahydro-ethylester (8.58%) and butanoic acid 2-(hydroxymethyl)-ethyl ester (5.43%). None of these molecules have a literature reported immunomodulatory activity. The molecule of with the highest abundance that has a literature reported immunomodulatory/antibacterial activity in ChemSpider (http://www.chemspider.com) is Itaconic acid diethyl ester (1.07%). The ability of LES Glutamax™ to protect against TNBS induced colitis in mice suggests that one or components are responsible for protection. The low abundance of the majority of features could indicate the presence of potent immunomodulatory molecules.

Table 6.2 presents non-volatile polar metabolites present in LES Glutamax™. This database was built using a targeted approach. There is little overlap between the underivatized and derivatized LES Glutamax™ data presented. Derivatized methodology allows otherwise non-volatile small molecules to be analysed by binding to a carrier molecule, trimethylsilane (TMS). This analysis was carried out at the Bio21 Institute, University of Melbourne, using a targeted approach; that is, only molecules for which standards have been run were identified as features of the chromatogram. A list of standards/targets is attached in Appendix C, and is a list of commonly encountered polar metabolites.

Combined, these two tables represent the major detectable components in LES Glutamax™. From the previous chapter I was able to establish that LES Glutamax™ is able to protect against TNBS-induced colitis. To my knowledge, this is the first time that the secretome of any helminth has been described using small molecule techniques. To contextualise the findings, those molecules for which KEGG compound numbers have been defined have been mapped against the predicted metabolic pathways of the human hookworm *N. americanus* (Figure 6.1).

To contextualise this data in terms of parasite metabolism and possible host-parasite interactions, I have mapped KEGG compounds to the biochemical pathway of *N. americanus* (Figure 6.1). Since *A. caninum* is one of the models for *N. americanus* it is a useful comparator for similarities. The *N. americanus* pathway is based on genomic sequence data [175] with the pathway enzymes being identified by homology to similar enzymes. The small molecules identified here are the substrates/products of these pathways.

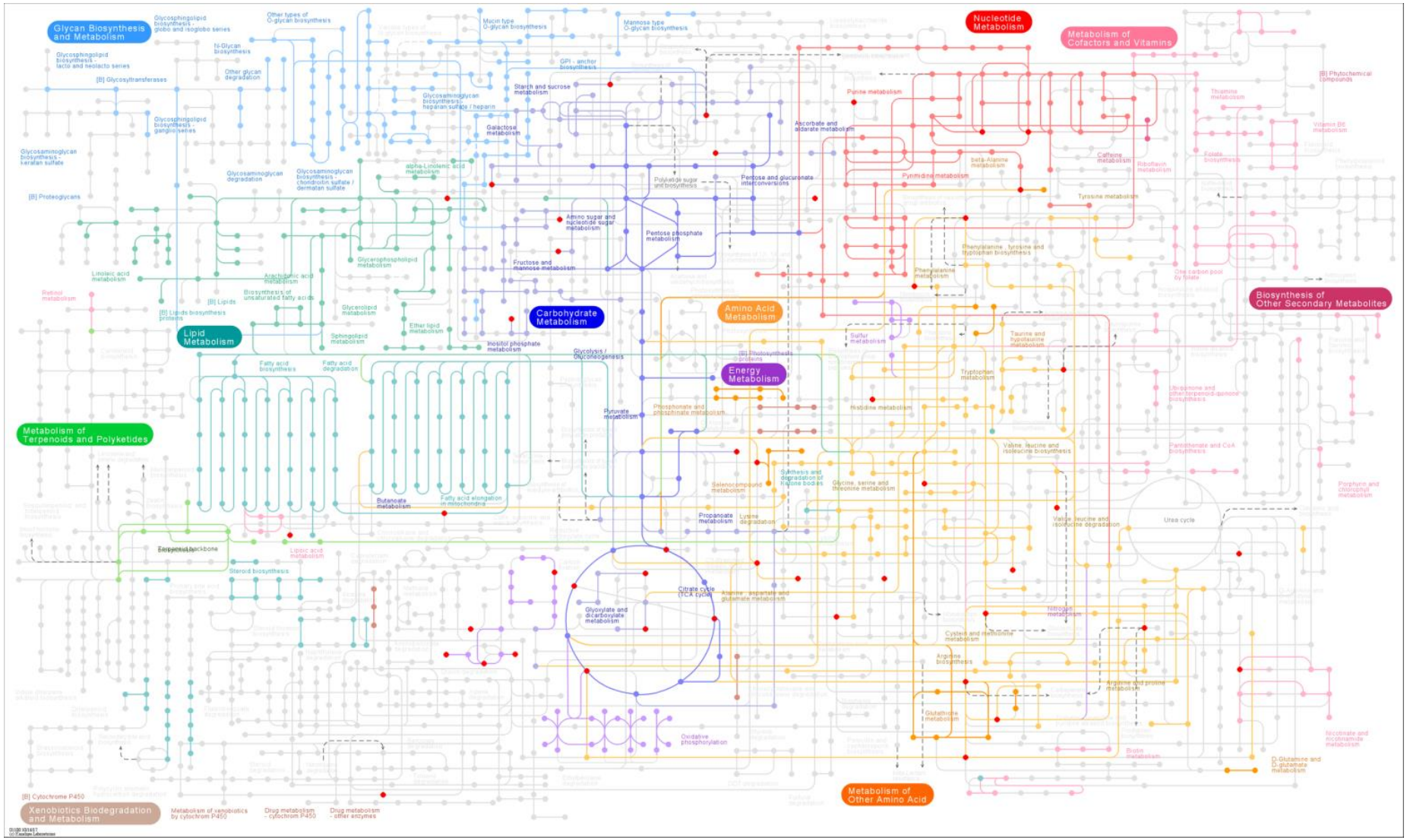


Figure 6.1. Small molecules from *A. caninum* LES Glutamax™ mapped against *N. americanus* biochemical pathways. Red dots are molecules isolated from *A. caninum* that are matched to known pathways from the *N. americanus* genome. Coloured lines represent putative pathways based on the genome of *N. americanus*. Greyscale pathways are those not yet described. Glycolysis pathways are poorly represented here, with the greatest representation of intermediates in the TCA cycle and amino acid synthesis pathways.

Mapped against *N. americanus* using the http://www.kegg.jp/kegg/tool/map_pathway1.html tool (organism code: nai, generated Oct, 2017)

6.4 Comparison of *A. caninum* LES Glutamax™ against LES Glucose database.

As established in the previous chapter, hookworms cultured in Glutamax™ produce LES that is consistently protective against TNBS induced chemical colitis in mice. In contrast, the LES produced by hookworms cultured in 2 g/L of glucose produce a significantly greater weight loss and poorer clinical outcomes in the TNBS bioactivity screen. This LES Glucose was significantly worse than the TNBS control and appeared to induce a pro-inflammatory response. Here, I compare the features of the CM LES extracts from both culture conditions to identify differences.

Figure 6.2 shows a heat map comparing all the features apparent in both LES CM Glutamax™ and LES CM Glucose (the CM extract of LES cultured on 2 g/L Glucose). This heat map reveals distinct differences between the two extracts.

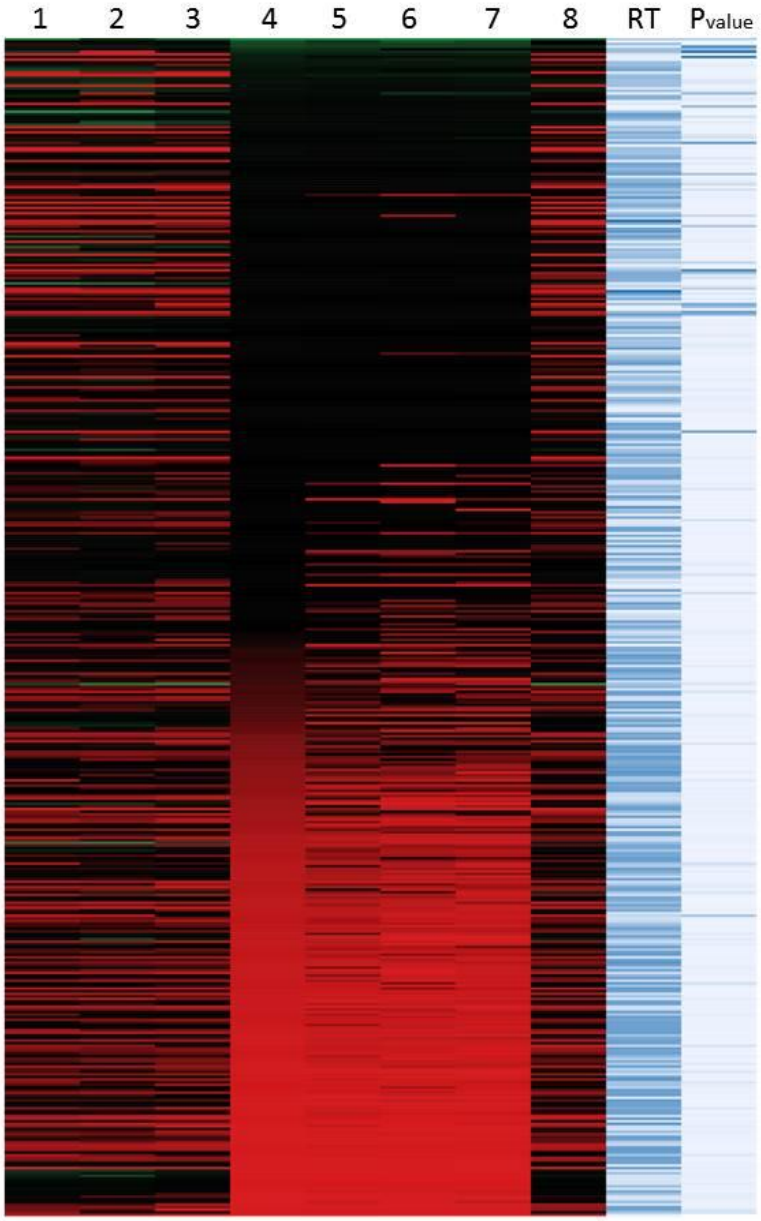


Figure 6.2. Comparison of GCMS features of chloroform: methanol extracts of LES Glutamax™ and LES Glucose reveal profound differences. Here the 437 detectable features of LES CM Glutamax™ (columns 4-7) are compared to those of LES CM Glucose (columns 1-3, 8) as determined by GCMS (Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer) (n=4). Features were compared using XCMS (https://xcmsonline.scripps.edu/) and reported with their retention time (RT) (ranked from lowest (pale) to highest (dark) and the P value (ranked from lowest (pale) to highest (dark). The P value expresses the significance of the difference between LES Glutamax™ and LES Glucose

A number of these features are significantly different between the two extracts; in Figure 6.3, all features that are significantly (* $P < 0.05$) different between the two samples are

visualised; 192 features were determined to be significantly different between the LES CM Glutamax™ and LES CM Glucose extracts.

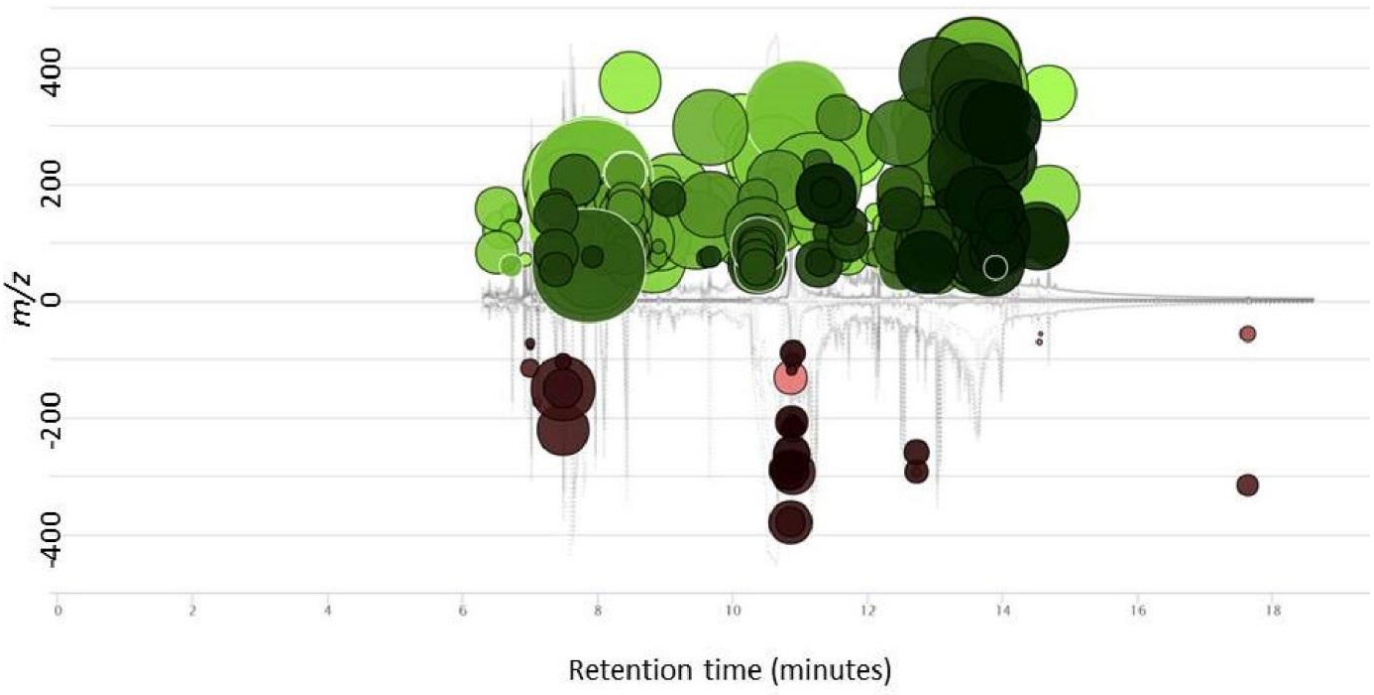


Figure 6.3. The comparison of GCMS features of chloroform: methanol extracts of LES Glutamax™ and LES Glucose reveals 192 significant features. This bubble plot shows the significant (P <0.05) differences between quantities of individual compounds in LES CM Glutamax™ compared to LES CM Glucose. Green bubbles represent an increase relative to LES Glucose with the bubble size representing the relative amount of each respective component; the deeper the shade of green, the larger the P value (more significantly different). Likewise, represented in red scale are those components that are reduced in LES CM Glutamax™ relation to LES CM Glucose, with the size of the bubble representing the relative amount, and the deeper shading indicating increased significance values. This graph was generated using untargeted GCMS data (Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer). Features were compared using XCMS (https://xcmsonline.scripps.edu/) [412]

It was also possible to directly compare the identified polar components in both data sets. Due to the constraints of XCMS it was not possible to compare these molecules alone and therefore this comparison was carried out in Graphpad prism to see if there were significant differences between LES CM Glutamax™ and LES CM Glucose. Figure 6.4 compares the polar compounds identified at the Bio21 Institute and highlights the significant differences between the extracts compared here.

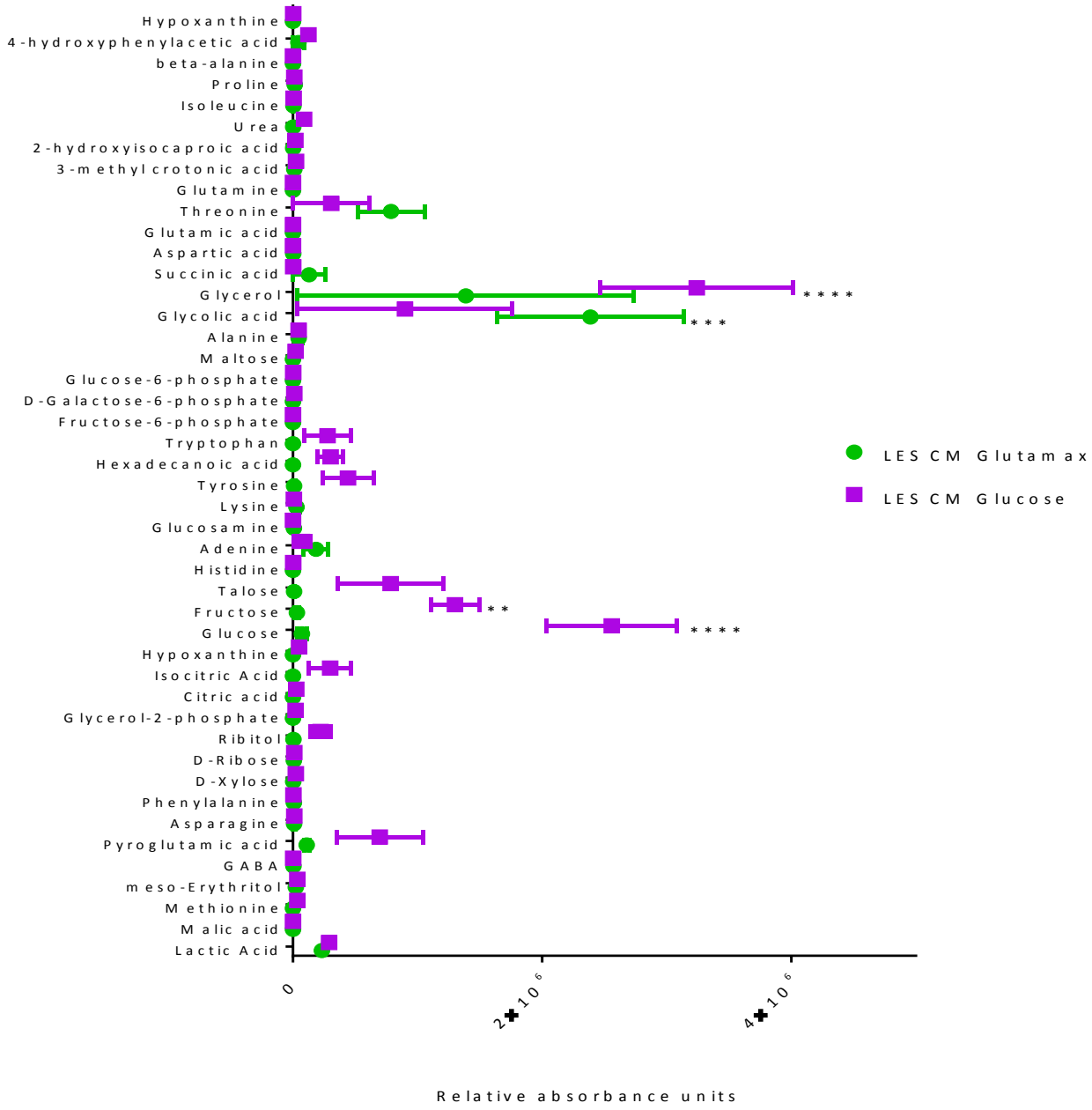


Figure 6.4. Comparison of non-volatile polar components of chloroform: methanol extracts of LES CM Glutamax™ and LES CM Glucose. There are significant differences in the amount of glucose (**** P <0.0001), fructose (** P = 0.0046), glycolic acid (*** P = 0.0003) and glycerol (**** P <0.0001) between LES CM Glutamax™ and LES CM Glucose. This plot was determined using targeted GCMS data (Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer).

6.5 Discussion

In this chapter the small molecule secretome of LES Glutamax™ is presented. One of the aims of this project was to identify small molecules that may have therapeutic value for the treatment of inflammatory disease. I have demonstrated in the previous chapter that treatment with LES Glutamax™ protects mice against TNBS-induced chemical colitis. A number of molecules with reported immunomodulatory activity were identified (Table 6.1), and one or more of these compounds may account for the protection observed in the TNBS bioactivity screen. Importantly, there are a number of unknown features identified in the LES Glutamax™ secretome, which may present as novel leads for therapeutic development. Additionally, it was possible to characterise the volatile polar compounds outlined in Table 2. Together, these provide a first pass characterisation of the small molecule ES component produced by the dog hookworm, *A. caninum*.

Only around 12% of the molecules described in Table 6.1 and Table 6.2 can be assigned KEGG numbers and can be used to annotate known hookworm biochemical pathways (Figure 6.1). These compounds can be assigned to the pathways of fatty acid synthesis, fatty acid elongation in the mitochondria, gylcerolphospholipid metabolism, or a partially represented tri-carboxcylic acid (TCA) cycle given the predicted genome sequence and reconstructed KEGG pathways. In the analysis carried out here there was no detectable oxaloacetate, cis-aconitate, 2-oxoglutarate, succinyl coA and fumarate in the TCA cycle pathway, the possible significance of this will be explored in the Discussion chapter. There were also molecules identified that could be assigned to carbon fixation, amino acid biosynthesis, carbon metabolism and 2-oxocarboxcylic acid metabolism, although representation of LES compounds in these pathways was sporadic and incomplete. Of the molecules that were assigned KEGG numbers, 19 were identified as ABC transporters (mineral and organic ion transporters); the significance of this is unknown.

The overwhelming majority of molecules identified in this study are novel. Of the 318 features identified in Table 6.1, a number could be eliminated on the basis that they are confounding agents (for example the silyl molecules are associated with GCMS column leaching). Yet others are from the supportive media. The Glutamax™ media contains PBS

and an anti-mycotic and antibiotic, the breakdown products of which account for some of the compounds identified herein. The breakdown products of both penicillin and streptomycin are poorly described so it is difficult to unpick their contribution to this dataset. The dimer glutamine-alanine was detected, and is the primary component of Glutamax™. Other molecules like DEET (insecticide) and phenobarbital (euthanasia drug) are presumed to be from the dog host and highlight the sensitivity of the GCMS method. It is also noteworthy that there is a high incidence of esters, possibly resulting from lyophilisation of phosphate buffered solutions [413].

The primary aim of this project was to identify molecules from *A. caninum* LES that may have therapeutic value. If the focus is to remain on drug discovery, the post-collection modification of small molecules is less important, as long as these collection procedures are reproducible and the molecules maintain protective bioactivity. However, this issue should be addressed if the primary interest is the molecular basis of host-parasite interactions. The removal of PBS could be achieved simply by running the LES collected through a preparative HPLC column to desalt the solution prior to lyophilisation.

During the course of this research it became evident that when hookworms were cultured in a media where the primary carbon source is glucose, the resulting LES generated very different bioactivity results compared to those of LES Glutamax™. From previous studies it was known that hookworms prefer not to metabolise glucose for energy but instead to divert it into amino acid production [406]. It possible to identify profound differences in the features of LES CM Glutamax™ (shown to be protective in TNBS colitis) and LES CM Glucose (Figures 6.2 and 6.3), with 192 features significantly different between the two culture methods. Using targeted GCMS it was possible to identify four molecules with significant differences between the two extracts. The possible implications of this will be discussed in the Discussion chapter.

Currently, proteaceous therapeutics targeting cytokines have serious potential side effects due to anti-protein responses. The ability to suppress inflammatory effects using small molecules is promising because it has the potential to overcome the limitations of modern IBD treatments. From the results above multicomponent fractions have the ability to suppress inflammatory effects of the TNBS colitis model. The identification of small

molecules that are stable, with targeted modes of action and relatively economical to manufacture would be the ultimate goal of future research. The findings here are an exciting step towards this goal.

6.6 The Next Chapter

Small molecules had not previously been screened by the Loukas laboratory. While other researchers had successfully used the TNBS colitis model for assessing anti-inflammatory small molecules [414] it was important to show that the model could be used as a bioactivity screen in our hands. The next chapter shows that the TNBS colitis model was able to highlight the anti-inflammatory potential of the small molecule; capnoidine.

Chapter 7

Proof of Concept

Identification of a small molecule therapeutic candidate using the TNBS colitis model

Preamble

This chapter I focus on the workup of the small molecule capnoidine as a therapeutic candidate using TNBS colitis as a bioactivity screen.

This work is important as it shows that in principle the TNBS colitis screen can be used down to the individual molecule level as a bioactivity screen for anti-inflammatory potential.

Here I investigated capnoidine as an agent to reduce inflammatory markers in the TNBS colitis screen and was able to show that it could protect against the key markers of chemical colitis.

This work validates the use of the TNBS chemical colitis model as a screen for small molecule bioactivity.

Out puts from this Chapter:

Capnoidine, an isoquinoline alkaloid of a medicinal plant, protects mice against acute experimental colitis. Shepherd C, Giacomin P, Miller C, Navarro S, Loukas A, and Wangchuk P.

PUBLISHED WORK Published here with permission.

Please note: This work has been edited for brevity.

Materials and Methods presented here are solely the work of P Wangchuk. All other laboratory work was performed by myself, C Shepherd and is included in the Materials and Methods (Chapter 3).

7.1 Introduction

Despite recent enthusiasm for the concept of computationally- and synthetically-derived drugs, there has been a decline in recent interest due to the relatively low number of drugs from these sources that have reached the marketplace [415]. Computationally-optimised drugs have relatively low chemical diversity compared with those sourced from natural products [28]. Natural products hold appeal for therapeutic drug discovery because they display superior chemical diversity and are considered evolutionarily optimised for biological function [28]. Long used herbal medicines have the potential to contain compounds that are beneficial [416]. Drugs such as aspirin, taxol, and vinblastine are all examples of currently used therapeutics derived from plants originating from traditional medicine [417-419]. Natural therapies, often known as complementary and alternative medicine (CAM), have the potential to be low cost and are well accepted by patients due to their "natural" origins. Patients with IBD rank among the highest users of CAM, with current or past use of CAM ranging from 21-60% [420-423]. Despite their increasing use by IBD patients, a lack of understanding of the bioactive chemical components and mechanisms of anti-inflammatory action are obstacles to the incorporation of many CAM or dietary treatments into mainstream medicine [392].

Recently, a systematic review of CAM treatments in IBD showed that 29 trials including phytotherapy, psychological therapy, acupuncture, and helminth therapy have been conducted to date [424]. Even though many studies showed promising results - particularly those involving poly-ingredient phytotherapy - most trials required additional substantiation [424]. Poly-ingredient herbal medicines are complex mixtures, so their mechanisms of action are, therefore, difficult to determine. Isolated bioactive molecules from medicinal plants or natural product compounds are, therefore, the ideal targets for drug discovery. Pre-clinical studies of medicinal plant extracts [414, 420, 425-428] have demonstrated anti-colitic properties. Similarly, crude extracts of the medicinal plants, *Corydalis dubia*, *Ajania nubigena* and *Pleurospermum amabile*, which are used in Bhutanese traditional medicine (BTM) as febrifuge and for treating infections related to inflammatory conditions [416], significantly reduced the production of TNF in LPS-activated human monocytic cells (THP-1) [429].

Following on from our previous work with crude extracts [429], we set out to isolate and test the anti-colitic properties of purified compounds from these medicinal plants. Of 25 compounds that were isolated and whose structures were determined from these three plant species [430-432], we assessed four of the most abundant compounds for their anti-colitic properties in a TNBS-mouse model of colitis. These four compounds (capnoidine (**1**), scoulerine (**2**) linalool oxide acetate (**3**) and isomyristicin (**4**) have not been explored for anticolitic potential until now. Since capnoidine (**1**) imparted the best protection of mice against colitis-induced weight loss, we conducted a detailed assessment of this compound as a novel lead therapeutic agent.

7.2 Results

Encouraged by the preliminary anti-inflammatory activities of the crude extracts of *C. dubia* (Figure 7.1a), *A. nubigena* (Figure 1b) and *P. amabile* (Figure 1c) against TNF in LPS-activated THP-1 monocytic cells [429], we isolated 25 compounds from these crude extracts as described previously[430-432].

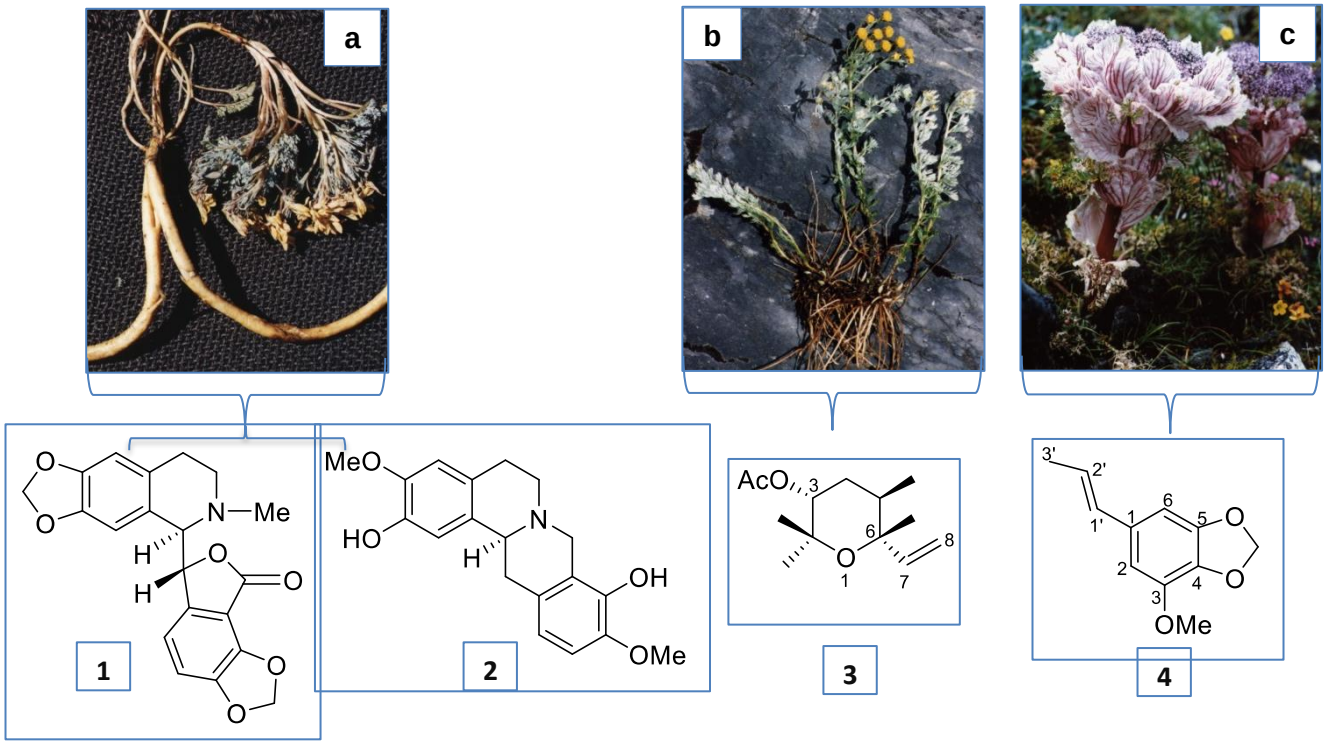


Figure 7.1. **The structures of the compounds isolated from three plant species and screened in the TNBS model of acute colitis**. Capnoidine (**1**) and scoulerine (**2**) were isolated from *Corydalis dubia* (**a**) [432], linalool oxide acetate (**3**) was isolated from *Ajania nubigena* (**b**) [430], and isomyristicin (**4**) was isolated from *Pleurospermum amabile* (**c**) [431].

Four of the compounds (Figure 7.1, **1-4**) were obtained in sufficient quantities to enable further assessment of their anti-inflammatory properties against TNBS-induced colitis in mice.

7.2 Preliminary assessment of compounds against TNBS-induced colitis

Body weight loss over time after administration of TNBS was monitored as a preliminary indicator of colitis (Figure7.2). Amongst the four compounds investigated, capnoidine (**1**) was the only one to confer protection of mice against TNBS-induced body weight loss (***P* = 0.0037; 2-way ANOVA). Linalool oxide acetate, isomyristicin and scoulerine did not confer any protection against weight loss compared with weight loss in mice given TNBS only. Further studies on the anti-colitic properties of these natural products were, therefore, restricted to capnoidine (**1**).

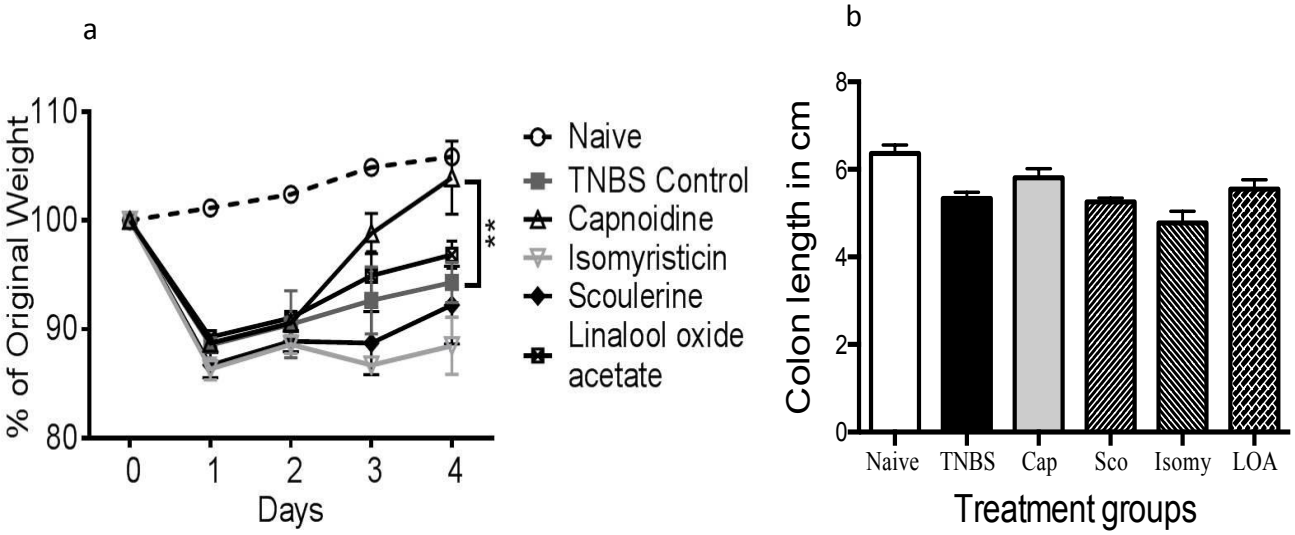


Figure 7.2. Levels of protection of mice conferred by four selected compounds (1-4) (50 µg/mouse dose) against the TNBS-induced ulcerative colitis. (**a**) Percent weight loss of mice. Capnoidine provided significant protection ((***P* = 0.0037; 2-way ANOVA) to the mice against the TNBS-induced percent weight loss compared with the TNBS control group that received vehicle alone. (**b**) Colon length of each group of mice. Although a trend in improved colon length was observed in a group of mice treated with capnoidine, it was not statistically significant. Error bars represent ± SEM. The results were pooled from two independent studies (total n = 10 mice) performed using 5 mice in each experiment.

7.3 Detailed investigation of capnoidine as a lead compound against TNBS-induced colitis

To examine the effect of capnoidine in more detail in the TNBS colitis model, the experiment was repeated with additional pathological and clinical parameters being measured. Mice were treated with capnoidine (50 μg/mouse) and then 4-6 hr later received intra-rectal administration of TNBS. Mice were monitored for their general wellbeing and clinical symptoms for three days. Confirming previous preliminary observations capnoidine-treated mice showed a highly significant reduction (****P = 0.0001) in body weight loss when compared with the TNBS only group (Figure 7.3a).

Capnoidine-treated mice also had significantly reduced clinical scores compared with TNBS only mice, including mobility (Figure 7.3b), piloerection (Figure 7.3c) and faecal consistency (Figure 7.3d). The overall clinical score of capnoidine-treated mice showed significant (**P = 0.006) improvement when assessed by 2-way ANOVA (Figure 7.3e).

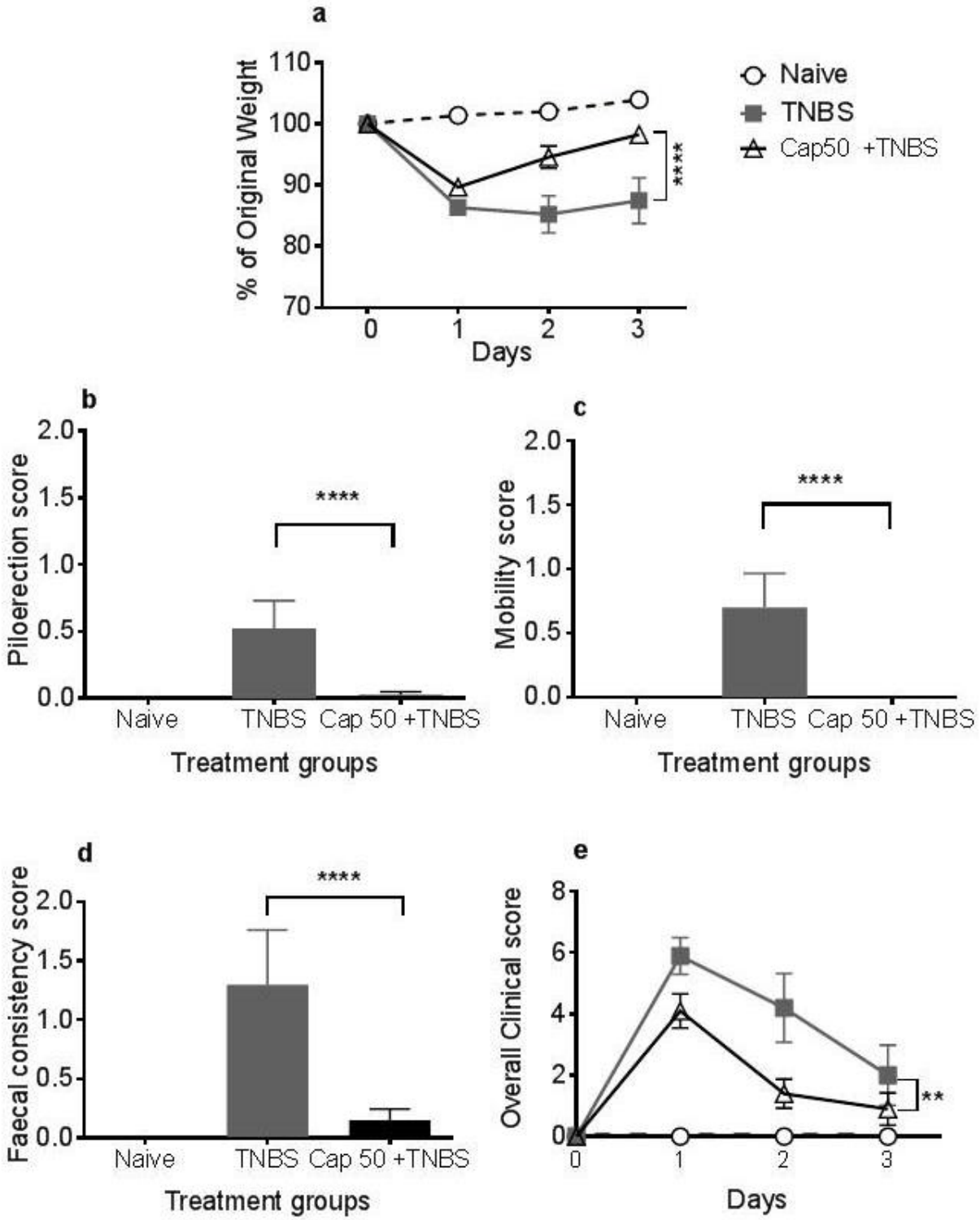


Figure 7.3. Capnoidine (Cap 50, 50 µg/mouse) treated mice showed significant improvements in clinical score compared with TNBS only mice. (**a**) Percent weight loss compared with original starting weight (****P = 0.0001), (**b**) Piloerection (****P = 0.0001), (**c**) Mobility (****P = 0.0001), (**d**) Faecal consistency (****P = 0.0001) and (**e**) overall clinical score (**P = 0.0060). Statistical analyses were performed by pooling data from groups of mice from two independent and reproducible experiments (Total n = 15). Error bars represent mean ± SEM. The results were pooled from two independent studies performed using 5 mice in each experiment.

7.4 Capnoidine treatment resulted in reduced intestinal pathology in mice

On day three post TNBS administration mice were euthanized and the colons were removed (Figure 7.4a). Colons were assessed for changes in macroscopic appearances including length, adhesions, colon wall thickening, mucosal oedema, ulceration and necrosis (Figure 7.4f). Compared with mice that were administered TNBS only, mice treated with 50 µg capnoidine had significantly longer colons (Figure7. 4b, $^{**}P = 0.0030$), and showed significantly reduced pathology scores (0-2 scoring matrix), including a reduced number of adhesions (Figure 7.4c, $^{**}P = 0.0055$), thinner walls (Figure 7.4d) ($^{*}P = 0.0112$), and less ulceration (Figure 4f, $^{**}P = 0.0032$). There was no significant difference in oedema between the two groups (Figure 7.4e).

Overall, the macroscopic pathology score was significantly reduced in capnoidine-treated mice compared with mice administered TNBS only (Figure 7.4g, $^{*}P = 0.0205$).

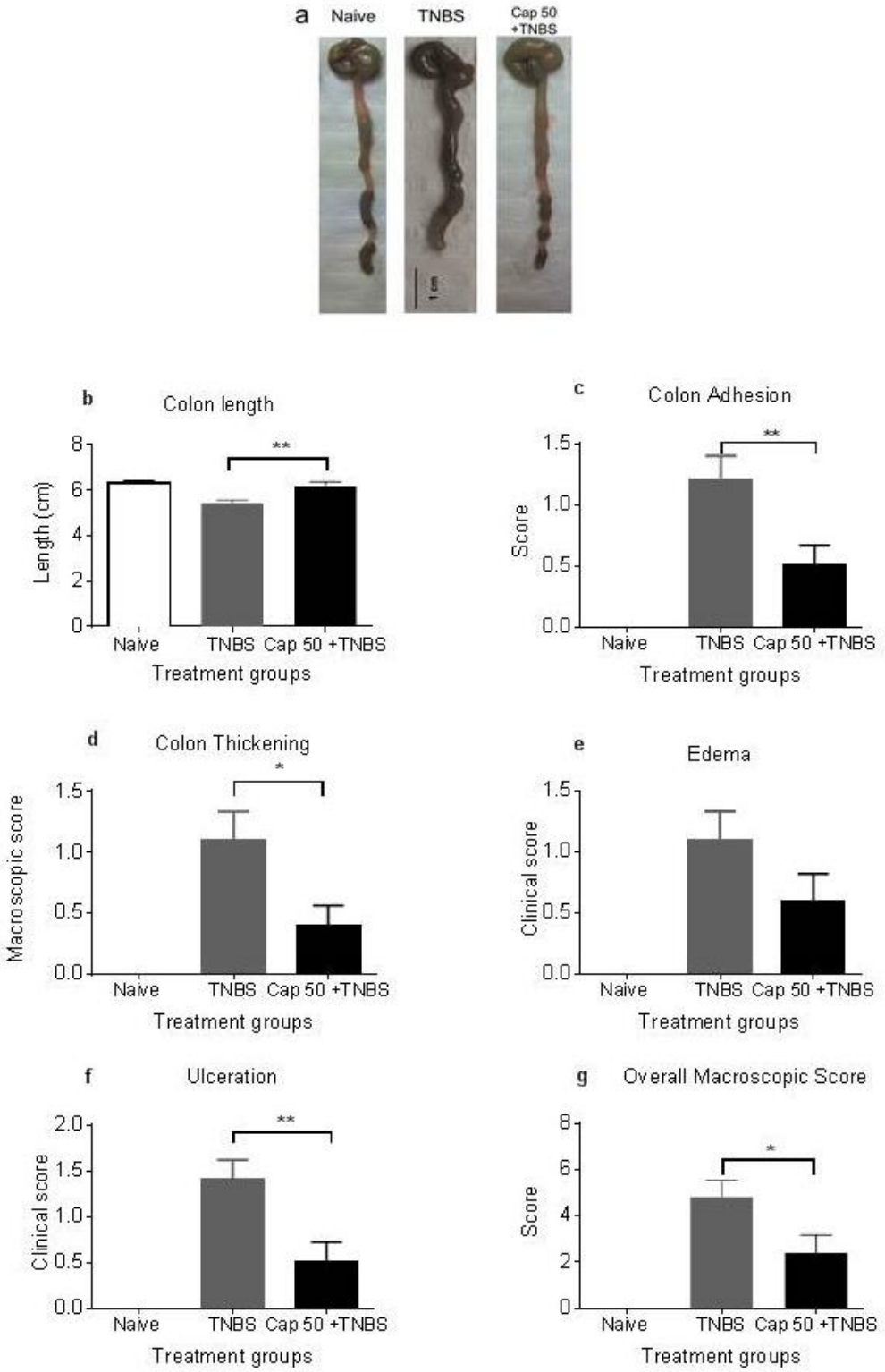


Figure 7.4. **Capnoidine (Cap 50 + TNBS) treated mice (50 µg/mouse) showed reduced colon pathology after administration of TNBS compared with mice given TNBS only**. Mice treated with capnoidine (Cap 50) presented with healthier looking colons (**a**) compared with mice treated with TNBS alone. (**b**) Colon length (***P* = 0.0030), (**c**) Adhesion of colon (***P* = 0.0055), (**d**) Colon wall thickening (***P* = 0.0112), (**e**) Oedema (n.s.), (**f**) Ulceration (***P* = 0.0032) and (**g**) Overall combined macroscopic pathology score (**P* = 0.0205). Statistical analyses were performed by pooling data from groups of mice from two independent but reproducible experiments (Total n=15 mice). Error bars represent mean ± SEM.

7.5 Capnoidine-treated mice had significantly reduced histopathology

The H&E-stained distal colon sections were observed for histological changes induced by TNBS administration and were scored as described by Hong *et al.*[392]. Untreated mice showed normal colon tissue architecture including gastric mucosa, lamina propria, epithelial compartments, goblet cell content, and colonic wall/musculature (Figure 7.5a).
Colons of TNBS only mice exhibited severe lesions and extensive histological damage, including thickening of the lamina propria and colon wall, evidence of oedema and mucosal erosion with destruction of healthy goblet cells (Figure 7.5b). In addition, increased numbers of leukocytes and polymorphonuclear and inflammatory cell infiltrates were evident in the lamina propria, intraepithelial compartments, and colon wall. Colons of capnoidine-treated mice showed no signs of acute colitis, including having healthy colon architecture with normal crypts and goblet cells, good mucosal integrity and no ulceration (Figure 5c). Scoring of histological sections for overall pathology showed that capnoidine-treated mice had significantly reduced histopathology (****P = 0.0001) compared with TNBS only mice (Figure 7.5d).

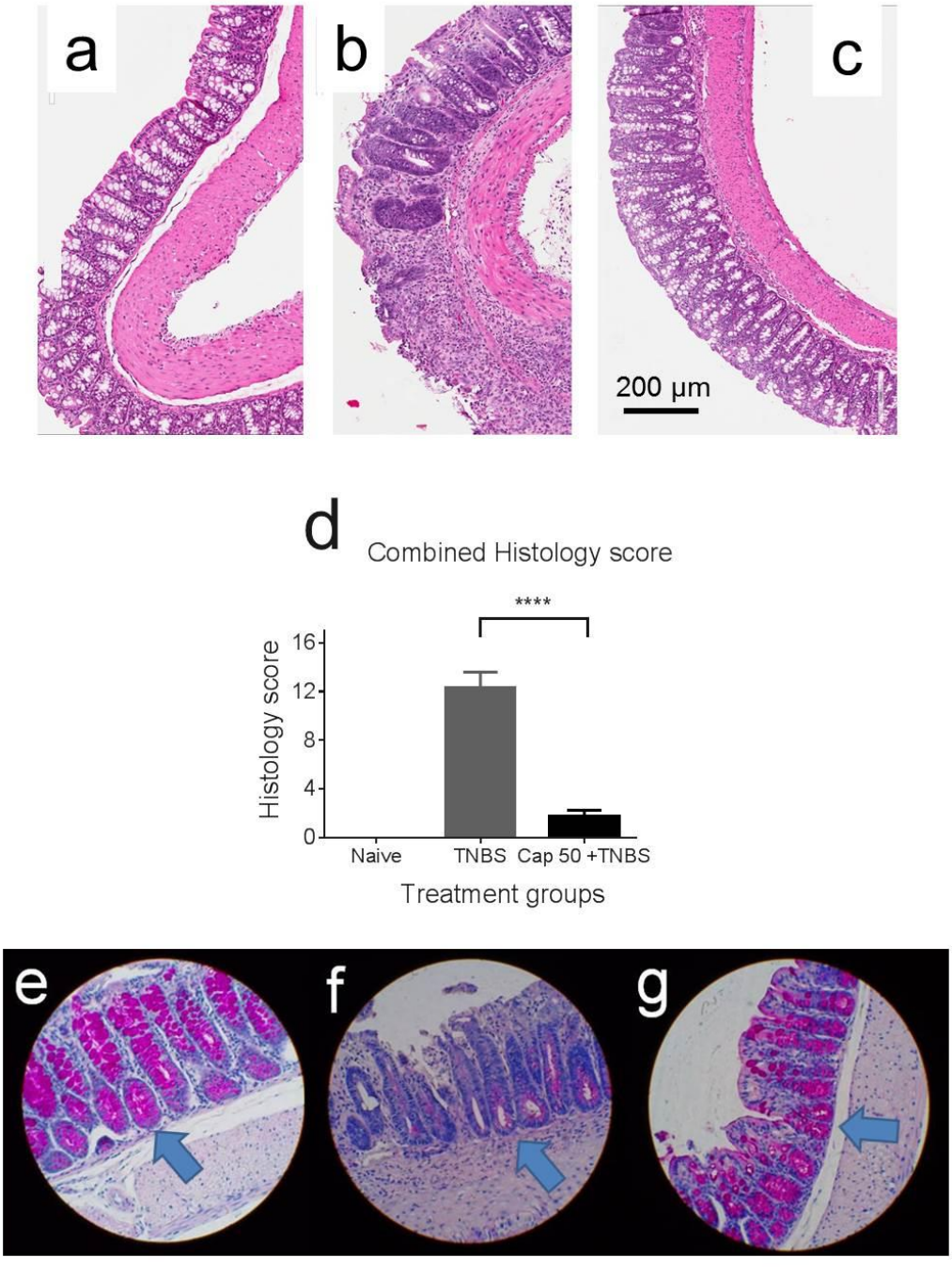


Figure 7.5. **Capnoidine-treated mice had significant protection against TNBS-induced histopathology. Representative photomicrographs of H&E stained colonic tissue sections.** (**a**) healthy naive control mice, (**b**) vehicle-treated TNBS treated mice, (**c**) Capnoidine-treated TNBS mice, (**d**) Blinded histological scoring of colonic pathology (****P = 0.0001). Statistical analyses were performed by pooling data from groups of mice from two independent but reproducible experiments (Total n=15 mice). Error bars represent mean ± SEM. Goblet cell destruction (as indicated by arrows) is demonstrated in Periodic acid–Schiff *(*PAS*)* staining of colon sections (**e**) healthy naive control mice, (**f**) vehicle-treated TNBS treated mice, (**g**) Capnoidine-treated TNBS mice

7.6 Cytokine responses indicate anticolitic effect of capnoidine

We next assessed the expression of the key cytokines IL-1β, IL-4, IL-5, IL-6, IL-13, IL-17A, TGF-β IFN-γ and TNF within the colons of mice following treatment with capnoidine. We also carried out RT-PCR on colons for cytokine IL-10 expression. Mice treated with capnoidine prior to administration of TNBS showed a significant reduction in the levels of IL-1β, IL-6, IL-13 (P values < 0.05) and TNF (P < 0.01), and an increase in IL-4 (P = 0.0355) in colon culture media when compared with the TNBS only group **(**Figure 7.6a-g**)**. There was no observable difference in IL-5 levels between treatment groups. There was an observable trend for reduced levels of IFN-γ in the capnoidine-treated group, but the difference was not statistically significant. Levels of IL-17A, TGF-β and IFN-γ in colon tissue homogenate (Figure 6h) of capnoidine-treated mice were lower than levels in TNBS only mice but the difference was not significant (P>0.05). We also determined the levels of the cytokine IL-10 RNA transcripts (Figure 7.7) however, there was no significant difference between IL-10 transcription levels .of the TNBS only and capnoidine treated mice.

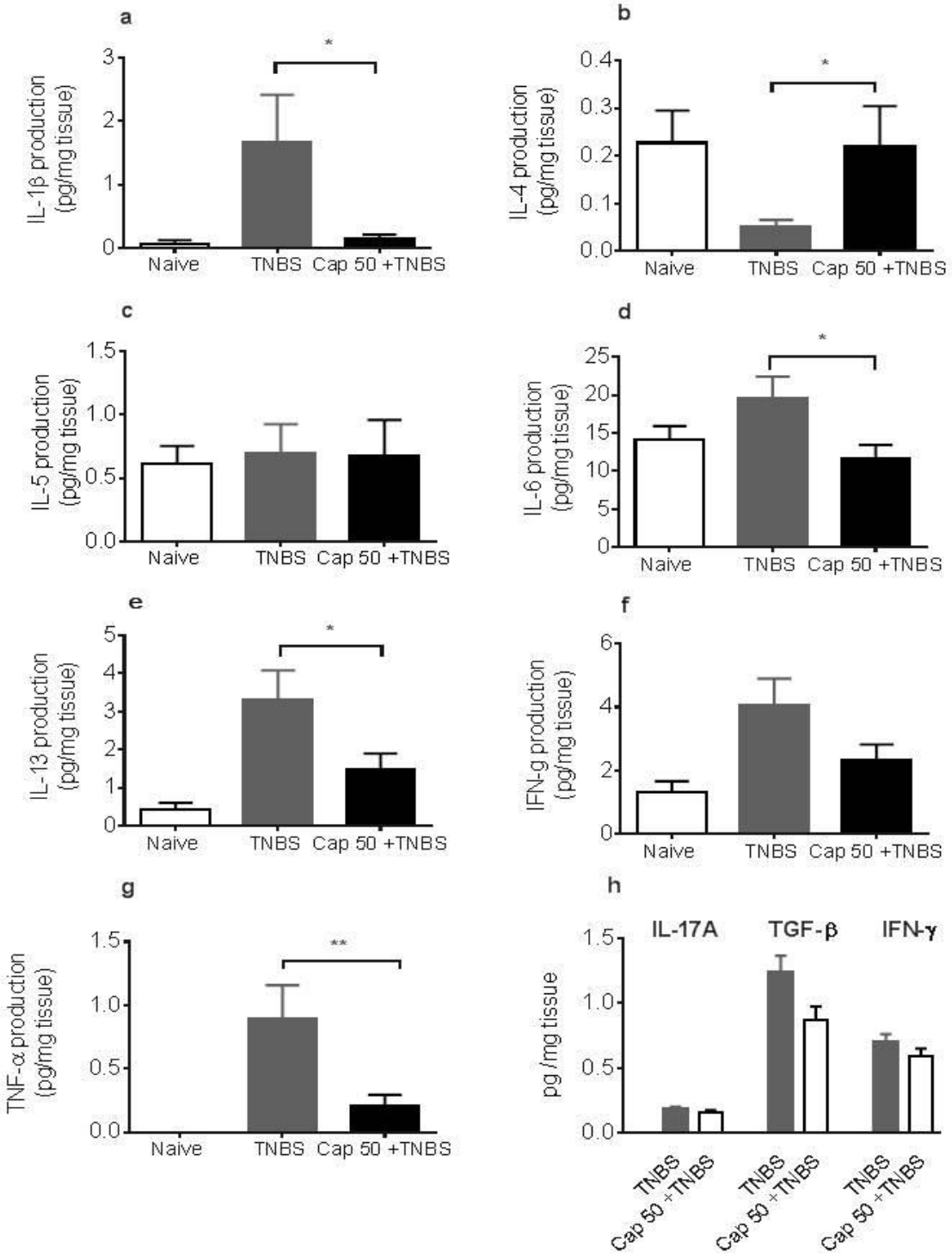


Figure 7.6. Capnoidine-treated mice have cytokine profiles that indicate protection against TNBS-induced colitis. Mice treated with capnoidine (Cap 50 + TNBS) showed cytokine profiles indicative of anti-colitic effects with significant reductions in levels of: (**a**) IL-1β (**P* =0.0441), (**d**) IL-6 (**P* =0.0413), (**e**) IL-13 (**P* =0.0462) and (**g**) TNF (***P* =0.0041) compared with levels in the TNBS only mice. There was an observable increase in the level of IL-4 (**P* =0.0355) (**b**) but there was no difference in the amount of IL-5 production (**c**) between the two groups. While there was a trend for a decrease in IFN-γ levels (**f**), this was not significant. A comparison of IL-17A, TGF-β and IFN-γ levels from colon homogenates (**h**) showed no statistically significant difference between TNBS only mice and

those treated with capnoidine + TNBS. Statistical analyses were performed by pooling data from groups of mice from two independent but reproducible experiments (Total n=15 mice). Error bars represent mean ± SEM.

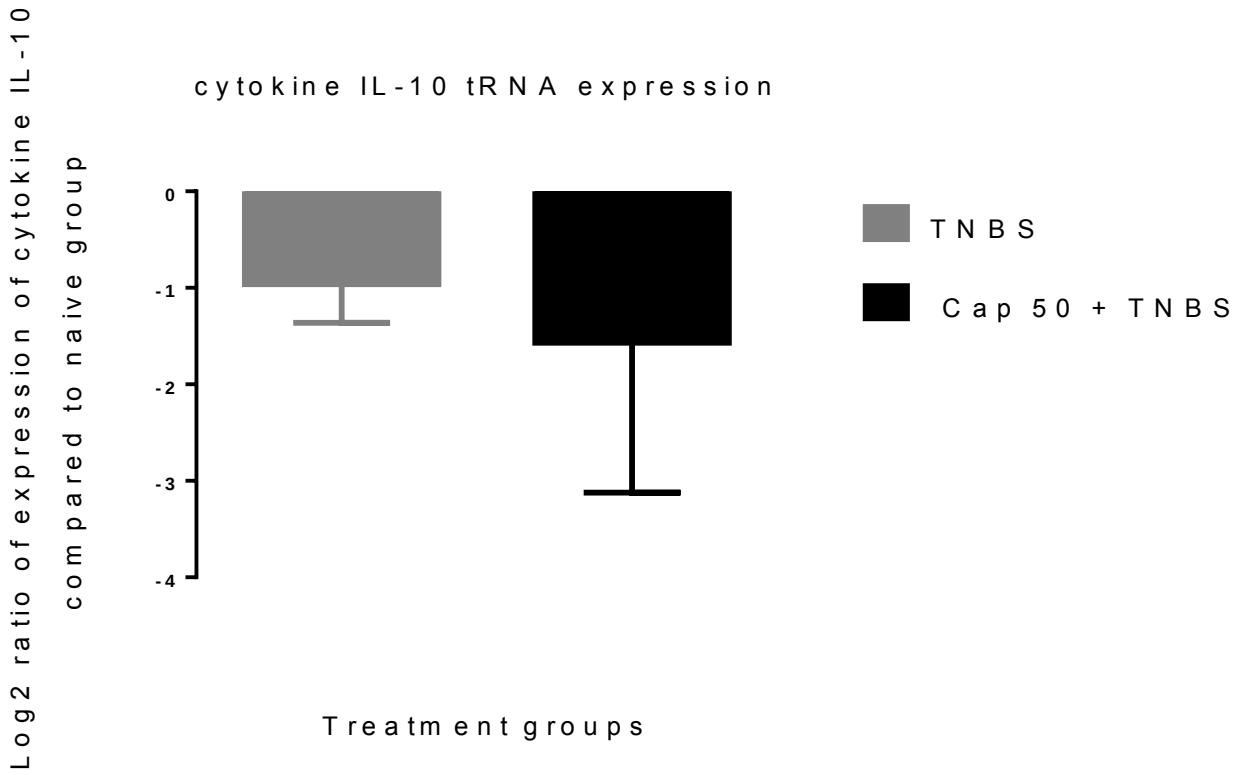


Figure 7.7 Capnoidine treatment does not significantly alter IL-10 tRNA levels when compared to TNBS only treated mice. Mice treated with capnoidine (Cap 50 + TNBS) showed tRNA levels that are not significantly different to TNBS only treated mice. PCR assays were performed in triplicate and assay efficiency for RT-PCR was determined to average 80-110% as calculated by LinRegPCR program. PCR was performed by pooling samples from groups of mice from two independent but reproducible experiments (Total n=15 mice). Error bars represent mean ± SEM.

7.7 Tissue p-IκB-α (Ser32) and p-NF-κB p65 (Ser536) levels of capnoidine treated mice were significantly reduced compared with TNBS treated mice

Molecules that are able to block the nuclear factor NF-κβ pathway signal transduction are attractive therapeutic targets in the treatment for IBD. This is because this signal pathway is thought to facilitate crosstalk between inflammatory pathways and promotion of intestinal inflammation associated pathology [433]. Transcription of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα is regulated through the phosphorylation status of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Translocation of

NFκB from the cytoplasm to the nucleus is, in turn, regulated by the phosphorylation status of inhibitor of κB-α (IκB-α). We determined the levels of p-IκB-α (Ser32) and p-NF-κB p65 (Ser536) in the colon tissue homogenates (Figure 7.8). Mice treated with capnoidine prior to administration of TNBS showed a significant reduction in the amounts of the p-IκB-α (Ser32) (P = 0.0259) and p-NF-κB p65 (Ser536) (P = 0.0466) respectively compared with TNBS only mice.

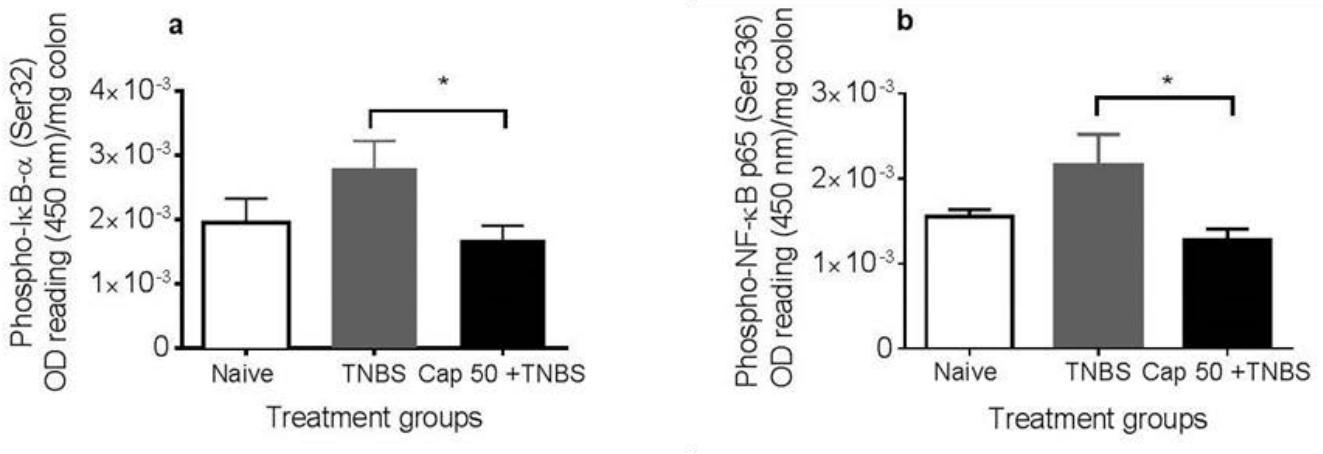


Figure 7.8. Levels of transcription factors (phosphorylation) in the colon tissue homogenates. Capnoidine-treated (Cap 50 + TNBS) mice have significantly lower levels of: (**a**) p-IκB-α (Ser32) (*P = 0.0259) and (**b**) p-NF-κB p65 (Ser536) (*P = 0.0466) compared with levels in TNBS only mice. Results were normalised to amount of colon tissue for each experiment. Statistical analyses were performed by pooling data from groups of mice from two independent but reproducible experiments (Total n=15 mice). Error bars represent ± SEM.

7.8 Discussion

The treatment regimen for IBD, especially UC, is limited and current therapies are associated with adverse side effects [434] and considerable socio-economic cost [435, 436]. Since the causes of IBD are complex, it is challenging to develop an appropriate treatment strategy. Current IBD drugs, especially the biologics, can have severe side effects [20], are expensive and difficult to administer [437]. As a result, a surge in interest in alternative medicine, especially naturally derived herbs and plant extracts, has emerged [438]. Nonetheless, the challenge with crude herbal extracts is the difficulty in elucidating mechanisms of action, and, therefore, emphasis is placed on isolation of bioactive chemical components as scaffolds for lead optimisation and therapeutic application. Due to their simple structures and anti-inflammatory properties [439], chemical entities including terpenoids, polyphenols, flavonoids and isoquinoline alkaloids have received attention as

alternative candidates for IBD therapy. These phytochemicals confer anti-inflammatory activity by reducing cytokine production and altering the expression of transcription factors associated with inflammatory pathways [440, 441]. For example, the quaternary coptisine, an alkaloid found in Chinese goldthread (*Coptis chinensis*), displays protection in colitis [442]. Similarly, noscapine, a phthalideisoquinoline alkaloid derived from *Papaver somniferum*, has exhibited anti-inflammatory activity through cytokine regulation [443] and this compound is currently under clinical trials for cancer therapy [444].

The genus *Corydalis* is not only well represented as a component of traditional medicines but also produces a number of small molecule isoquinoline alkaloids that display anti-inflammatory properties [445]. Interestingly, the isoquinolone alkaloids from *Corydalis* plants that display anti-inflammatory activity show little structural similarity [445, 446]. This reflects the difficulty in establishing Structure Activity Relationships (SAR) between phytochemicals and mechanisms of suppressing inflammation [447].

In this study, we have investigated the anti-colitic properties of 4 compounds (Figure 7.1), which were isolated from *C. dubia, A. nubigena* and *P. amabile* - in a mouse model of acute TNBS colitis. TNBS-induced colitis shares many clinical, histological and biochemical features with human UC and is a widely utilised *in vivo* protocol for screening of anti-colitic compounds [6, 426]. Questions arise as to whether an acute TNBS-induced model could perfectly represent a human IBD, which is chronic inflammatory in nature. It must be acknowledged that human IBD - CD and UC- while chronic, are well documented to be episodic in nature. The aetiologies of both acute and chronic intestinal inflammatory disease is often enigmatic and are often linked to prior acute inflammatory disease incited by pathogens including viruses, bacteria, parasites, dysregulation of the intestinal immune response, or autoimmune disorders. Presently, there is no 'perfect' animal model that can address all the mechanisms involved in intestinal inflammation of IBD. Each animal model has an array of advantages and disadvantages to its use and therefore a comprehensive study examining multiple aspects of intestinal inflammation requires multiple models. We are aware of the relative strengths and limitations of each model of colitis used to study acute and chronic inflammatory diseases of the mammalian intestine, and the scope and relevance of outcomes achievable based on this knowledge. The ability to induce inflammation to mimic common human diseases is an important factor of a successful animal model and a review by Jiminez *et al*. [448] highlighted the suitability of TNBS as an

incitant of both acute and chronic inflammation. TNBS elicits cell-mediated mixed Th1 and Th2 cytokine-based immune responses and induces transmural inflammation in the gut with clinical, morphological and histopathological features similar to those of human IBD. Clinically, IBD is characterized by severe diarrhoea, bleeding, abdominal pain, fluid and electrolyte loss, which underlines the initial acute nature of IBD. Similarly, patients with ulcerative colitis experience significant colonic thickening and dense infiltration of neutrophils, monocytes, macrophages and T cells typically on the mucosal layer of the bowel wall. All these clinical and histological signs are exhibited in the TNBS colitis model, which we have employed here for screening the anti-colitis properties of four compounds isolated from three Bhutanese medicinal plants. These three medicinal plants, which grow in the high mountain ranges of the Himalayas in Bhutan, have been used in BTM for hundreds of years for treating various diseases including symptoms related to inflammation. Of the four compounds tested, capnoidine (**1**) (isolated from *C. dubia*) showed the best and significant anticolitic properties. We have demonstrated here that treatment of mice with capnoidine prior to administration of TNBS resulted in a reduction in severity of clinical symptoms (Figure 7.3) accompanied by a reduction in colon pathology (Figure 4) and histopathology (Figure 7.5). This was associated with a significant reduction in the production of IL-1β, IL-6, IL-13 and TNF (Figure 7.6), which are the signature cytokines of human UC. The ability of this compound to significantly reduce expression of TNF in the colon of mice after TNBS administration supports previous observations on its parent *C. dubia* crude extract [429]. Indeed, TNF is a major target of monoclonal antibody-based biologics for treating IBD and other inflammatory diseases, including rheumatoid arthritis and psoriasis [437, 449-451]. Generally, the production of IL-1β and TNF is associated with the severity of disease in human UC, possibly due to persistent activation of NF-*k*B [452]. Capnoidine treatment resulted in significantly reduced levels of IL-6 in the colon compared with TNBS only mice. IL-6 is a multi-functional cytokine produced by a wide range of immune cells, and plays a role in the differentiation of Th17 cells. IL-6 is highly expressed in flaring IBD, and is, therefore, a target for monoclonal antibody-based treatment of IBD patients who failed to respond to anti-TNF therapy [453].

Transcription of pro-inflammatory cytokines is regulated through the activities of NFκB[49]. Our results showed significantly lower levels of phosphorylated IκB and phosphorylated NFκB in mice pre-treated with capnoidine compared with mice given TNBS only (Figure 7.8)

suggesting that capnoidine is able to reduce cytokine production possibly via inactivation of the NFκB pathway. However, the precise mode of action of capnoidine remains unknown. Our study was curtailed by the amount of capnoidine available, a common limitation encountered when working with natural products and validation of ethnomedicines [441]. Capnoidine treatment also suppressed expression of the Th2 cytokine, IL-13, which has been attributed to driving pathogenesis in UC and is the focus of efforts to develop new biologics [454]. On the other hand, capnoidine treatment resulted in increased expression of the Th2 cytokine IL-4 when compared with levels in the TNBS only group, resulting in similar levels to those found in healthy control animals, and likely reflected the absence of inflammation in the gut. The cytokine IL-10 is implicated as an anti-inflammatory cytokine in colitis [455] however we found no significant difference in tRNA levels between TNBS only and capnoidine treated mice. Thus, thorough investigation of capnoidine highlighted its highly significant anticolitic properties including protection of mice against clinical signs (bodyweight loss, diarrhoea, and piloerection), pathological features (colon shortening, adhesion, thickening, oedema and ulceration), dysregulated cytokine production (decreased TNF, IL-1β, IL-6 and IL-13, and elevated IL-4), and histological changes (mucosal layer, goblet cells, lamina propria, and infiltration of inflammatory leukocytes and polymorphonuclear cells).

While the exact mechanism of action remains unknown, the ability of capnoidine to significantly reduce the inflammasome signalling pathway 1 molecules implicates the possible disruption of inflammasome formation and the prevention of the inflammatory process. The ability of capnoidine to protect mice against these hallmark features of murine colitis, which are translatable clinical symptoms and pathology in human UC, is a strong indication that this compound is a potential candidate as a new anticolitic compound. This study also shows that capnoidine is at least one of the bioactive components that are responsible for the anti-inflammatory properties of its parent crude extracts of *C. dubia*. *C. dubia* has been harvested historically for the treatment of symptoms and diseases that bear relevance to inflammatory conditions. Our findings highlighted the value of exploring traditionally used herbal medicines as a source of novel anti-IBD therapeutics.

Capnoidine is a simple small molecule that shares close structural similarity with noscapine (Figure 7.9), which is currently used as an antitussive and antimitotic drug in many countries and is also in clinical trials for treating many cancers[444]. Like noscapine, capnoidine (logP

= 3.38) also met the criteria of the 'Lipinski Rule of 5[24], which predicts the drug-likeliness of a compound.

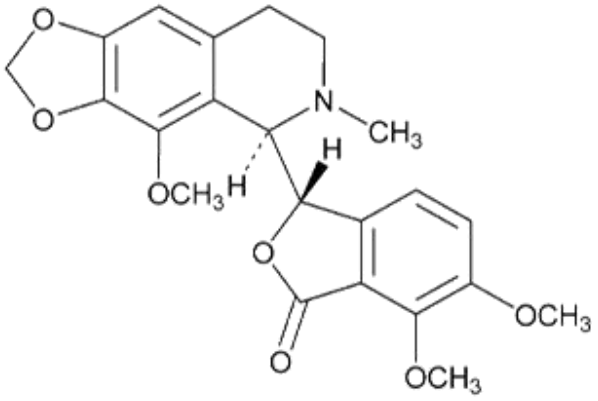


Figure 7.9. **Noscapine structure has scaffold similar to capnoidine (1)**

In addition, our previous cytotoxicity study of capnoidine against normal Vero cells (kidney epithelial cells of African green monkey, *Cecopithecus aethiops*) found that it is non-toxic [432]. These chemical and biological properties of capnoidine underscore its potential as a lead scaffold upon which to develop newly derivatized anticolitic small molecule drugs and opens up new avenues for other therapeutic studies and applications. With further future medicinal chemistry works on lead optimisation, derivatization, appropriate dose response analysis, cytotoxicity, bioavailability, stability and mechanism of action of capnoidine; this compound, as a natural product anti-inflammatory agent, presents potential to inspire new treatment strategies in an area of care where mainstream medication often fails or emancipates major side effects.

7.9 Materials and methods

These methods were performed by P Wangchuk and included here for completeness. Essentially, they describe the methods used to extract and identify the capnoidine molecule prior to testing in the colitis model. All other work as described in the results was performed by C Shepherd.

Plant materials

The selected plant materials – *Corydalis dubia* (Fumariaceae, herbarium voucher specimen number (HVSN) 78)), *Ajania nubigena* (Asteraceae, HVSN 73), *Pleurospermum amabile* (Umbelliferae, HVSN 29) – were collected from the Himalayan mountains of Lingzhi in Bhutan in July-August 2009 and the herbarium specimens were preserved at Menjong Sorig Pharmaceuticals (MSP), Ministry of Health, Thimphu, Bhutan. Although the whole plant of *C. dubia* is used in BTM, only the aerial parts of *A. nubigena* and *P. amabile* were used in this study. We collected those parts of plants from their natural habitats and dried them in the shade/sun in accordance with prescribed BTM traditional methods of drying.

7.10 Preparation of compounds for the TNBS experiment

The extraction, fractionation, isolation and characterisation of compounds from these three plant species were reported earlier[430-432]. Briefly, the air-dried plant materials (2 kg) were chopped into small pieces and were repeatedly extracted with analytical grade or HPLC grade methanol (MeOH) (5 x 3 L over 48 h). The extracts were filtered and then concentrated using a rotary evaporator at 35-50°C to produce the crude MeOH extracts of each of the three medicinal plants. The MeOH extracts were dissolved in MeOH:H_2O (200 mL, 1:9), then fractionated with different solvents (3 × 100 mL) including hexane, CH_2Cl_2, $CHCl_3$, EtOAc, *n*-BuOH to obtain the respective solvent extracts, and purified repeatedly using flash column chromatography packed with Merck Kieselgel 60 PF_{254}. Aluminium-backed silica plates (0.2 mm silica thickness, Merck, normal and reverse phase) were used for separating isolates of smaller quantities. Solid compounds were crystallized from a mixed solvent system of chloroform/methanol. UV light (short wavelength of 254 nm, long wavelength of 366 nm) was used for visualization of the separated bands on TLC. Dragendorff's reagent was used for visualising alkaloids on TLC plates. Ceric ammonium molybdate solution was used for staining non-alkaloid compounds spotted on TLC plates. A Reichert hot-stage apparatus was used for determining the melting point of the compounds and was not corrected. IR spectra were obtained on neat samples using a Smart Omni-Sampler Avator ESP Nicolet spectrometer. While LR-ESI-MS of the compounds were obtained using Micromass Waters Platform LCZ (single quadrupole, MeOH as solvent), the LR-EI-MS and HR-ESI-MS were acquired using Shimadzu GCMS-QP-5050 (DIT at 70 eV) and Micromass Waters Q-ToF Ultima (quadrupole time-of-flight) mass spectrometers,

respectively. The GC-FID was obtained using a Shimadzu GC-2010 Plus gas chromatograph with hydrogen as carrier gas (1.5 mL/min at 40 °C in a constant total flow mode) and the GC-MS spectra was acquired using Shimadzu QP5050A GC-MS system (electron impact (EI) mode at 70 eV) with helium as a carrier gas. Separation was achieved using a Restek fused silica capillary column (Rxi-5MS: 30 m × 0.25 mm i.d., 0.25 µm film thickness). The compounds were identified by comparing their mass spectra with the NIST and NISTREP mass spectra library of GC-MS data system and further confirmed by comparing the NMR chemical shifts of the spectra. ^{1}H-NMR, gCOSY, ^{13}C-NMR, APT, gHMBC, gHSQC, TOCSY and gNOESY spectra of the compounds were acquired by a 500 MHz Varian Unity Inova, 500 MHz Varian Premium Shield (VNMRS PS 54), and 300 MHz Varian Mercury spectrometers (in deuterated solvents, CD_3OD or $CDCl_3$)[21-23]. Other chemicals and solvents used for natural product isolation and characterisation were described previously [430-432].

Twenty-five compounds in total were isolated from these three plant species. We selected - on the basis of abundance - four major compounds (**1**-**4,** Figure 1) for investigation of their anti-colitic properties in a mouse model of TNBS colitis. As described previously [432], capnoidine (**1**) (8 mg) and scoulerine (**2**) (9.4 mg) along with other six isoquinoline alkaloids were obtained from the crude MeOH extract of *C. dubia* [432]. Capnoidine (**1**) was obtained as colourless crystals and had a molecular weight (Mol. Wt.) of 367 and the formula $C_{20}H_{17}NO_6$. Scoulerine (**2**) was isolated as a reddish brown solid with a Mol. Wt. of 327 and the formula $C_{19}H_{21}NO_4$. Linalool oxide acetate (**3**) (1.2 g), along with 7 other compounds, was isolated as colorless oil (Mol. Wt. 212, formula $C_{12}H_{20}O_3$) from the crude MeOH extract and essential oil of *A. nubigena* [430]. Isomyristicin (**4**) (185.3 mg), along with 9 other compounds, was isolated as colorless oil (with Mol. Wt. 192 and the formula $C_{11}H_{12}O_3$) from the MeOH extract and essential oil of *P. amabile* [431]. Stock solutions of these four compounds (**1**-**4**) were prepared by dissolving one milligram of each of compound in 1% DMSO/PBS (0.22 µm filtered) to obtain 1 mg/mL stock concentrations. Each mouse was administered 50 µg of this stock solution.

Chapter 8
Discussion

Preamble

In this chapter, I bring together my research findings and discuss:

- The value of the TNBS colitis model as a bioactivity screen for anti-inflammatory small molecules, and its successful application herein using a range of complex mixtures down to individual molecules.
- The possible therapeutic value of these molecules is explored in the context of our search for novel anti-inflammatories.
- The possible roles of small molecules produced by hookworms in host-parasite interactions.
- The small molecule milieu characterised herein and how it fits with our understanding of parasite metabolomics.

Finally, I will look at the limitations and the questions raised by this study. I will touch on future research directions that my research presents and avenues of endeavour that may be explored.

8.1 Long friendships run deep

For eons humans have collected, foraged and harvested medicinal herbs. They have climbed precarious peaks, waited for the right season and picked at just the right stage. They have processed, stored, and administered in particular ways. It turns out that this is for good reason, with a significant proportion of modern drugs being sourced from ethnobotanicals [28]. These traditional medicines through their long associations have proved a rich source of drug discovery. However, in the last 50 years there has been a dramatic increase in inflammatory disease such as asthma, diabetes, multiple sclerosis and inflammatory bowel diseases in modernised populations [37]. These modern diseases are chronic, seemingly have no cure and the modern drug cabinet appears to be bare for treatments. New medicines are clearly needed for these ailments.

The search for drugs to treat inflammatory diseases has cast a wide net but is restricted by our lack of understanding of the diseases themselves and the limitations of modern drug discovery. Breakthrough medications such as the anti-TNF biologics are expensive, have serious complications and patients can be resistant to treatment [16, 451]. Turning back to long-term and natural biological associations seems a rational approach to modern drug discovery [456]. The association of human and hookworm is one of the most ancient and enduring. Parasites, as outlined by the “Old Friend’s Theory” may be integral to human immune system maturation [38], with hookworms being one of the most co-dependent of these relationships [280].

The human hookworm, in its gut dwelling stage, can evade ejection by the host for up to a decade. And, as evidenced by worm burdens in adults, the host fails to develop any sort of meaningful protective immunity against this parasite [50]. This evasion is an active process; parasites produce molecules that interact with the host immune system to prevent their expulsion, but at the same time, immune effector mechanisms maintain worm burdens at levels that the host can usually tolerate. The ancient association of human and hookworm has meant that the human immune system has evolved to expect the input of these molecules. It is speculated that the lack of exposure to pathogen (and particularly helminth) molecules may be one of the underlying causes of inflammatory disease. So what are these molecules? How do they interact with the immune system? Moreover, can they be used to

treat inflammatory disease? All of these questions are difficult to answer in the setting of the human hookworm. Study is ethically constrained and there are restrictions on the usefulness of many animal model equivalents.

The dog hookworm is one of the models studied to understand the human-hookworm relationship. Proteomic study of the ES products produced by the gut dwelling adult hookworm has revealed a number of proteins that are immunomodulatory. For example, the 41 kDa protein Neutrophil Inhibitory Factor (NIF) prevents the adhesion and aggregation of neutrophils and ultimately prevents their activation [162]. In addition, homologues of tissue inhibitors of metalloproteases (TIMPs) have been shown to prevent dendritic cell activation [246] and have been successfully used to treat inflammatory disease in mouse models [164, 457]. There is also evidence of hookworms being able to induce crosstalk between the innate and adaptive immune system by inducing the production of IL-4 [256]. In an apparent theme of parasitism, evasion of the host is no one-trick pony, with multiple proteins (and possibly other molecules) acting at different sites in different ways [39, 329].

There has been very little work carried out on the small molecules that hookworms produce within their suite of ES molecules. This low molecular weight ES complement (LES) and how it contributes to host tolerance has not been explored [39]. In addition, there has been no investigation of the therapeutic potential of LES from any helminth, despite small molecules being a much more attractive proposition than peptides or proteins from a therapeutic standpoint due to their potential ease of administration and increased stability and bioavailability.

8.2 A small molecule bioactivity screen: Is the TNBS chemical colitis model suitable?

One of the difficulties of studying the immunomodulatory effects of small molecules is that the targets can be either poorly defined or extraordinarily narrow, and therefore it is difficult to screen a broad range of components. What is apparent is that small molecule interactions can occur in surprising ways and there are literally catalogues full of small molecules that interact with the immune system [458]. Since there was no existing literature to guide the selection of a screening model for helminth products, I have used the TNBS chemical colitis mouse model as a broad-based bioactivity screen for anti-

inflammatory activity. This model is known to induce Th1/Th17 type inflammation with Th2 hyperplasia, as well as activation of the innate inflammatory processes. The TNBS model has the additional advantages of only needing minimal amounts of screening material and having a relatively short experimental window.

While the TNBS mouse colitis model was well established in our laboratory [5], it had never been used to screen small molecules for anti-inflammatory activity in this setting. To validate the TNBS colitis model, I administered the small molecule, capnoidine, to mice (50 μg/mouse) and assessed its protection against TNBS induced colitis. Capnoidine is a small molecule sourced from plant material and extracted in part using small molecule techniques that were proposed for use in this project with hookworm LES. I was able to show reduced clinical, macroscopic, and histological disease in capnoidine-treated mice compared to the TNBS only treated mice. I was also able to show that there was a decrease in the inflammation-associated cytokines IL-1β, IL-6, IL-13 and TNF in capnoidine treated mice. This series of experiments was able to establish that small molecules can be screened successfully using the TNBS colitis model.

8.3 Small molecules from *A. caninum* ES can protect against TNBS chemical colitis – LES Glutamax™ is complex

In a number of experiments presented in this body of work, it is possible to show that the low molecular weight component produced by adult *A. caninum* can protect against TNBS-induced colitis. Treating mice with LES Glutamax™ (50 μg/mouse) prior to administration of the TNBS chemical confers significant protection against the clinical, histological and macroscopic parameters of TNBS chemical colitis [31]. Cytokine responses of mice pre-treated with LES Glutamax™ revealed a significant reduction in the pro-inflammatory associated cytokines IL-1β, IL-13, and TNF. However, there was an increase in the levels of INF-γ and IL-17A, which are associated with inflammation. This is the first time it has been demonstrated that hookworm LES has immunomodulatory potential.

Characterisation of LES Glutamax™ by GCMS revealed over 300 features. Of these features, only a handful were identified and attributed immunomodulatory activity (Table 6.1) based on prior reports in the literature. From a therapeutic discovery standpoint, these results are encouraging. The molecules present in *A. caninum* LES (*Ac* LES) have not been defined in the

context of their anti-inflammatory activity and a number of undescribed molecules have been identified. Given that many of the current drugs used to treat IBD rely on their ability to reduce TNF levels, the suppression of TNF by *Ac* LES is encouraging. Due to the complexity of LES Glutamax™, separation of components using small molecule techniques was carried out.

8.4 Small molecule techniques – purifying therapeutic candidates from crude *Ac* LES

A small molecule technique is a term used to describe the organic chemistry methods used to separate molecules based on their physical and chemical properties. They are a commonly utilised approach in ethnobotanical research. The research described herein is the first report of such techniques being applied to helminth ES components. Using a simple extraction method - the Folch extraction [400] - it was possible to separate LES Glutamax™ into three components. A polar salt extract, which showed no protection in the TNBS bioactivity screen, chloroform: methanol extract and an ethyl acetate extract.

Both the LES Glutamax™ chloroform: methanol (LES CM Glutamax™) and the ethyl acetate (LES EA Glutamax™) fractions were protective in the TNBS colitis screen. It was decided that only the LES CM Glutamax™ fraction would be pursued in this study since it had the greatest therapeutic potential. LES CM Glutamax™ prevented the onset of inflammation in the TNBS chemical colitis model. However, of the cytokines assessed in this model, only IL-1β was significantly suppressed when compared to the TNBS control. Because of the strong protection against weight loss, low clinical disease scores, and preservation of histological structure, I decided to further investigate LES CM Glutamax™.

Using a preparative thin layer chromatography plate, LES CM Glutamax™ was separated into 11 bands that were extracted and tested in the TNBS bioactivity screen. Of these bands, only four showed comprehensive protection against weight loss, clinical presentation, and macroscopic score. Analysis of colon cytokines from mice treated with these four bands in the TNBS colitis model revealed distinct differences. Band 4 induced significantly lower IL-1β, IL-13, INF-γ and TNF levels when compared to the TNBS only treated mice. As a result, this band presents as the most promising for further investigation and has the highest

therapeutic potential. Identifying the individual components of band 4 is beyond the scope of this thesis, but this work is ongoing in our lab.

It is possible to demonstrate here that small molecule techniques can be used systematically to identify molecules of therapeutic interest when guided by a suitable bioactivity screen.

8.5 LES Glutamax™ - small molecule description – big picture implications

The characterisation of the LES produced by *A. caninum* represents the first time that the small molecule secretome has been described for any helminth. Analyses reveal a complex mix of components, many of which have not been annotated. It was possible to map 43 of these molecules to the putative biochemical pathways of the human hookworm, *N. americanus*. The observations here agree with what is already known about parasite metabolism.

A common pattern in parasite metabolism is a functionally incomplete TCA cycle [459, 460], which is what we observe here. It is also known that parasites rely on the host for glucose and subsequently use this in anaerobic glycolysis rather than classical glycolysis [407]. Parasitic helminths are known to favour lipids and amino acids over carbohydrates as a source of energy [461]. These parasites utilise the TCA cycle and fatty acid degradation for amino acid metabolism and not for ATP production [462]. Of the molecules identified herein, the majority map to the TCA cycle, amino acid synthesis, and fatty acid metabolism. It has been noted by others that successful parasites without exception utilise anaerobic metabolism during active parasitism. The advantages of this metabolism are three-fold: the end products are short chain fatty acids which are well established as anti-inflammatory agents; short chain fatty acids are molecules produced by commensal bacteria and may aid to mask the presence of hookworms; finally, the actual metabolomics pattern may signal to surrounding cells a general “health and well-being” pattern.

It is intriguing that these 43 molecules are readily detectable in the secretome of hookworms, as opposed to just their somatic tissues. Parasites are known to be fermentive metabolisers; that is the end products of metabolism are molecules like short chain fatty acids. Yet here, the cellular intermediates of biochemical pathways are easily discernible. There is an argument to be made that these molecules may be released from dying cells or

the worm itself, but given the relative impermeability of the hookworm's outer cuticle, I propose that the hookworm is actively secreting these molecules to reinforce an environment of tolerance and immune homeostasis around its site of attachment.

There has been much recent interest in immunometabolism - the metabolism profiles of activated immune cells - and profiles that are associated with immune homeostasis [463]. There are six pathways that are strongly associated with immune function; glycolysis (pro-inflammatory), the TCA cycle, the pentose phosphate pathway, fatty acid oxidation, fatty acid synthesis and amino acid metabolism. For example, high levels of fatty acids and amino acids can inhibit activation of the mTOR pathway in cells, and is considered anti-inflammatory [463]. It would be interesting to know the levels of mTOR expression at the site of hookworm attachment in the gut to see if this is one of the mechanisms of immune tolerance. It is also known that M1 and M2 macrophages have distinct differences in their TCA cycles; M2 macrophages have a complete TCA cycle whereas M1 macrophages have a TCA cycle that is broken in two places (after citrate and succinate) [463]. Noteworthy is that M2 macrophages are associated with helminth expulsion, yet we see here that *A. caninum* has adopted the broken TCA cycle pattern associated with M1 macrophages.

It is notable that hookworms when cultured in 2 g/L of glucose produce a LES that drives pro-inflammatory responses in mice, as evidenced by the TNBS bioactivity screen (Figure 5.1). It is well established that glycolysis is employed by many activated immune cells; in macrophages glucose utilisation drives the production of IFN-γ and inflammatory processes [463]. The results herein suggest that other molecules associated with glucose metabolism may help to drive or reinforce inflammatory processes. It may be possible by comparison with the protective LES CM Glutamax™ to determine what these features may be.

8.6 Limitations and Future work

The primary limitation of this study was supply and access to dog hookworm for culture and study. Access to cadaver dogs for the harvesting of hookworms can be restricted on an ethical and social level, and this proved to be a significant barrier for this project. Raising dogs in an experimental setting for experimental infection and harvesting of hookworms was not an appropriate option for this project for ethical, logistical, and financial reasons. Hookworm infections in dogs display a distinct seasonality in its pattern. During the course

of this study, I found that high- to medium-intensity hookworm infections in dogs occurred in the May-June period coinciding with the last rains of the monsoon season. Harvests outside of this time invariably failed to yield appreciable numbers of hookworms; one collection event memorably gave a total of 50 hookworms from a total of eight dogs. While in the latter stages of this study, we were able to negotiate access to cadavers supplied to the vet school, which allowed for a more consistent supply of hookworm material to continue studies. This required travel to Townsville from Cairns to collect material and transport it back to the laboratory and all the complications that entails.

In the introduction of this thesis, evidence was presented that helminths actively produce proteins that interact with the host immune system to induce immune tolerance. The themes of immunomodulation show that helminths have produced a complex array of proteins that could not be predicted by bioinformatics alone. These ICH are pleiotropic but act to induce modified Th2 response in the host [39]. The findings here suggest that the protein component produced by helminths is not the complete story of immunomodulation by helminths. There are small molecules are present in the ES that are associated with intracellular processes yet they are found in relatively abundant quantities. If viewed through an immunometabolism lens these molecules may well prove to be a strategy by *A. caninum* to provide a further layer to the immunomodulatory environment around the site of attachment by molecular signalling to epithelial cells, macrophages and DCs that the "status quo" is being maintained. By bathing the area in molecules that indicate immune homeostasis it may be possible for the helminth to evade expulsion. Not only do these molecules act as anti-inflammatory signals to surrounding cells but also helminths are known to produce molecules that are foodstuffs for commensal bacteria of the gut. By adopting this strategy it promotes an immune-tolerogenic environment by proxy. To this researcher it would not be surprising to find that in addition these helminths are producing a number of small molecules that directly disrupt inflammatory processes like the formation of inflammasome. One possible experimental technique to explore these questions could be the application of NMR-metabolomics, where the metabolomic profiles of individual cells can be profiled. It may be possible to discern the immune-metabolomic profile of buccal cells and the cells of the host at the site of the hookworm attachment.

Curtailed access to material meant that projects such as re-investigating peptide components using modified collection techniques was not possible. With the increasing sensitivity of new mass spectrometry technologies, these limitations may be countered to some degree. Moreover, the ability to perform *in situ* proteomic MS/MS analysis of tissues and cellular level NMR metabolomics means that some of the interesting questions around the roles of hookworm small molecules in host-parasite interactions that were raised as a result of this thesis might be answered. Are peptides involved in host-parasite interactions? Are exosomes involved in small molecule cross talk? Do hookworms produce molecules that block pro-inflammatory metabolism/pathways? Do hookworm small molecules directly interact with host immune cells? Is it possible for these molecules to be developed as treatments for inflammatory disease?

This project has hinted at both the biological and therapeutic potential of the small molecules present in LES. However, for any molecule identified to garner interest for therapeutic development establishing anti-inflammatory results in more than one animal model is desirable [464]. Ultimately, identifying small molecules for the treatment of human disease requires the transition to human models. One way to do this is cell proliferation studies of human PBMCs. Cells are labelled with CFSE and incubated in the presence of the small molecule of interest to assess activation via proliferation of human lymphocytes *in vitro* [465]. Using this method would be possible to identify cells are activated by the small molecule of interest. Another technique that could be valuable it that once the identity of specific small molecule candidates is carried out binding assays to human arrays could be performed. It is important to do this, as many examples of encouraging results in mouse models do not translate into clinical trial efficacy. This can be due to the differences between murine and human immune systems. One way to mitigate this is the use of humanised mice models as suggested above.

Finally, there are many features highlighted in LES Glutamax™ that have never before been described; they are either completely unknown or have no recorded bioactivity; this presents itself as an exciting field for therapeutic discovery. I have been able to demonstrate reproducible protection with hookworm LES in the TNBS chemical colitis model, and using small molecule techniques, shown that it is possible to narrow the focus to a small number of candidate molecules that suppress inflammation and associated production of pro-

inflammatory cytokines. While the mechanism or the exact molecule(s) by which protection is induced at this stage is not known it is exciting to think that hookworms are producing a small molecule secretome that may enhance host tolerance. As mentioned previously, final characterisation of the bioactive compounds is ongoing, with the ultimate aim of identifying suitable therapeutic candidates.

8.7 Final Summary

I have established that the small molecule complement produced by the dog hookworm *A. caninum* has anti-inflammatory activity. Through small molecule purification techniques, I have isolated molecules that fall within the therapeutic window.

In addition to this, I have characterised using GCMS the small molecule secretome of *A. caninum*. This is the first time that a helminth has been described in this way. From my results, I speculate that hookworms employ similar strategies to suppress inflammation at the small molecule level as they do at the protein level. Hookworms likely produce multiple small molecules with distinct anti-inflammatory mechanisms of action, and these compounds probably act in synergy to induce immune tolerance and protect the worm against expulsion from its mammalian host.

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Appendix A

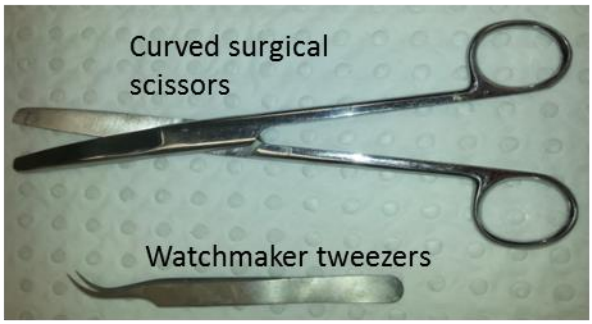
The preparation of Excretory/Secretory component from intestinal parasites of dogs.

Aim: To describe the culture of the hookworm *Ancylostoma caninum* from dogs. This procedure can be adapted for other intestinal parasites as listed at the end of the document.

Scope: This procedure covers the harvesting of intestines from the dog to the preparation of ES material from parasites for transport.

Equipment Requirements:

- CO_2 (5%) Incubator at 37°C
- Waterbath (37°C) for warming substrates
- Laminar Flow Cabinet
- Centrifuge –50 ml tubes/1000g/10min/4°C
- Large plastic tray
- Curved surgical scissors
- Watchmaker tweezers
- Pipette boy
- Scalpel



Consumables and reagents:

- Petri dishes (25 mL capacity)
- 50 mL Falcon tubes
- Pipette boy 25 mL tips
- 5x Substrate – Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies) + 5% antimycotic/antibiotic + 1% Glutamax. i.e. 500 mL DPBS + 25 ml anti/anti + 5 ml Glutamax
- 2x Substrate – Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies) + 2% antimycotic/antibiotic + 1% Glutamax. i.e. 500 mL DPBS + 10 ml anti/anti + 5 ml Glutamax

Substrate is prepared aseptically and can be stored at 4 °C prior to use. Warm substrate in a 37°C waterbath before to use. For every 50 hookworms approximately 75 ml of 2x Substrate will be required for a 3 day culture.

Collection of Intestines

Hookworms are obtained from the small intestine of cadaver dogs. Intestines should be harvested from freshly euthanized animals that are suspected of hookworm infestation (this can be confirmed by faecal examination for hookworm eggs).

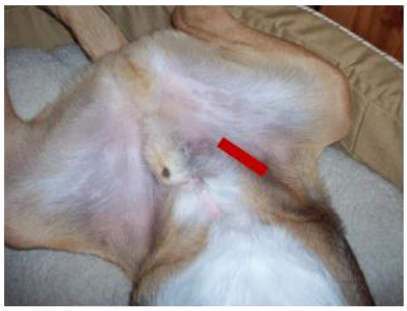
Handy Hint: Hookworms will detach from the gut as the tissue cools which will make it difficult to identify worms. Processing intestines quickly is essential to maintain Integrity of the parasite.

Hookworm ova in a faecal specimen

Safety: Dog parasites can cause zoonotic infections in humans . We recommend full PPE including gown, gloves and surgical mask. In addition plastic apron and sleeve protectors are advised.

Removal of the small intestine from dogs

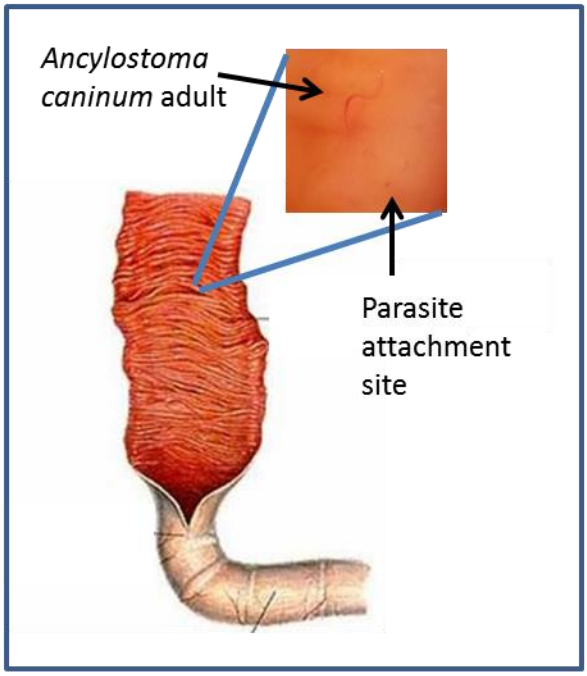
1. Using a scalpel make a small transverse cut on the lower abdomen of the animal. Cut through the dermis and the abdominal lining carefully to expose a small amount of the intestine.
2. Using a finger, loop under the intestine and gently pull the intestine through this hole. Locate the junction to either the large intestine or stomach. Cut the intestine at this point and gently pull the small intestine out easing it away from the mesenteric membranes until the other end is reached. Tie each end of the intestine to prevent content loss. Place in a container for transport to the laboratory.
3. Dispose of the animal as approved.



Approximate location of incision

Hookworm removal and processing

1. Process intestines over a large plastic tray.
2. Using curved surgical scissors cut down the length of the intestine to expose the inner surface.
3. Using clockmaker tweezers gently pick the hookworms from the intestine and transfer to 5x substrate (minimum 25mL). Avoid any faecal material and intestinal tissue .
4. Centre the worms by swirling the container for several seconds. While the worms are centred use a pipette boy with a 25 ml tip to suck as much of the liquid as possible without sucking up the worms. Discard the substrate.
5. Quickly add more warm 5x substrate to the container.
6. Repeat steps 4 to 5 at least 4 times (more if needed) to remove any visible intestinal matter. Centre the worms then using a new 25 ml tip suck up all the worms making sure to also suck up as little liquid as possible. Place approximately **50** worms per Petri dish.
7. Transfer worms to a petri dish and add 25 mL of 5x substrate. Incubate for 2 hours at 37°C in a CO_2 incubator.



Handy Hint: Anchoring the tissue as shown below aids in exposing the hookworms and also keeps the tissue warm (worms will detach as they cool)

Small worms that are NOT attached to gut wall may be pinworm and should not be collected.

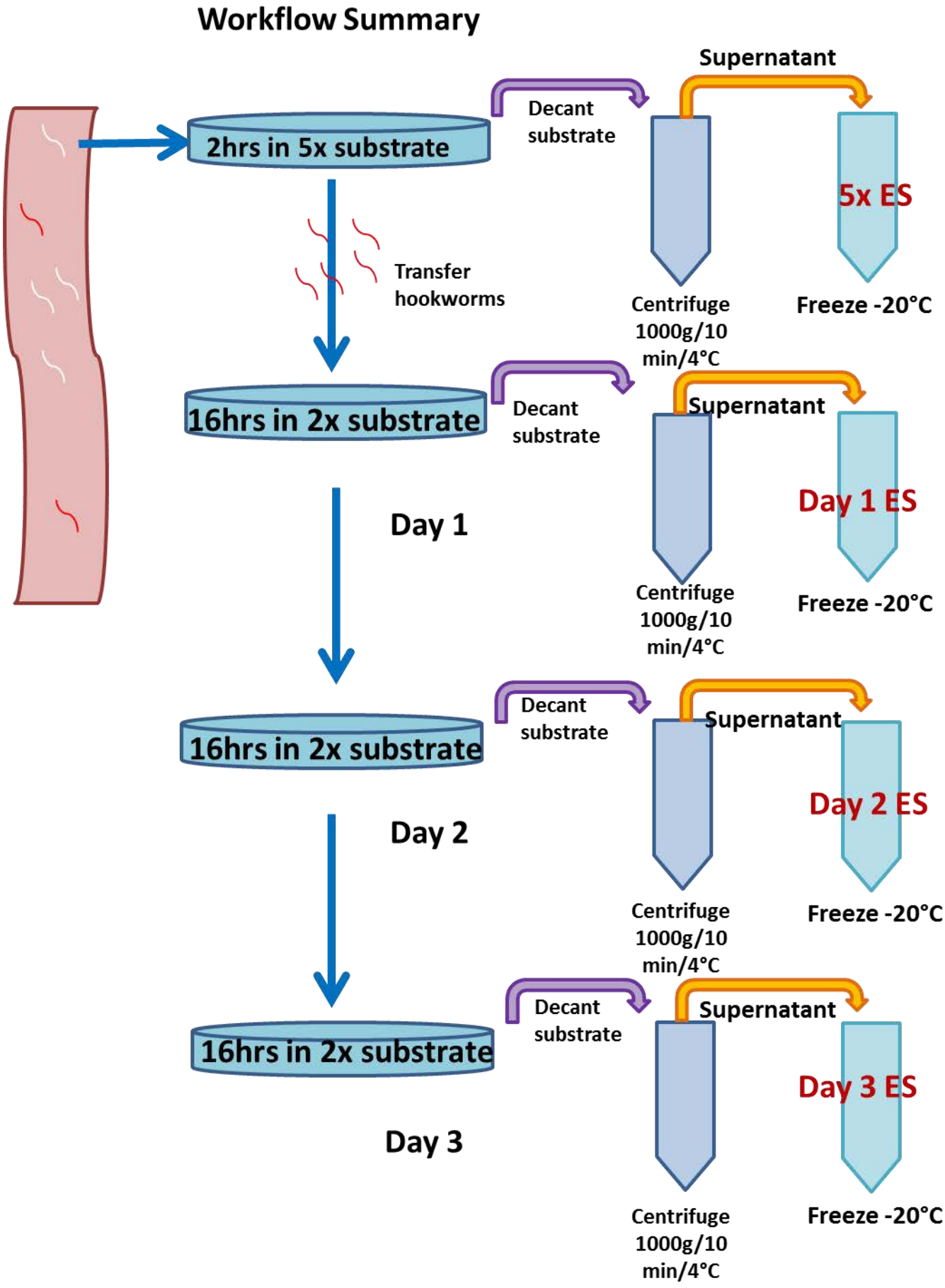
Safety: Dog parasites can cause zoonotic infections in humans . We recommend full PPE including gown, gloves and surgical mask. In addition plastic apron and sleeve protectors are advised.

ES Production from hookworms (all work to be carried out in laminar flow cabinet)

1. During the 2 hour incubation period clean all equipment and wipe down the laminar flow hood with ethanol. *This incubation period is important as it allows the 5x anti/anti component to neutralise the bacteria present and the hookworms to purge their gut contents.*
2. Remove petri dishes from incubator. Centre the worms by swirling petri dish then using a new 25 ml tip suck up all the worms making sure that you suck up as little media as possible. Transfer to a new petri dish containing 2x substrate.
3. Retain the 5x substrate and process as per step 7-8. Label these tubes clearly as **5x ES** with the date. Store at -20C or -80C until needed.
4. Wash the hookworms 3 times as done previously but with 2x substrate.
5. Incubate worms in approximately 25ml of 2x substrate for 16 hours in a 37°C CO_2 incubator (This is **Day 1** ES collection material).
6. After 16 hour incubation collect substrate (contains ES proteins) using a 25 ml tip and adding to a 50 ml falcon tube (you can pool more than one petri dish into a falcon tube).
7. Centrifuge at 1000g 10 min at 4°C to pellet the worm eggs.
8. Transfer the supernatant to a new tube – you can pool but only up to 40 ml (to allow for expansion in the freezer). *Label tube clearly with day of ES collection and date.* Store at -20C or -80C until needed.
9. Repeat steps 4 to 7 for **Day 2** and **Day 3** collections. **Remove dead worms with tweezers between each incubation** (these worms will show signs of disintegration or will not be moving in the substrate – nudge motionless worms with the tweezers- they should move if alive). Store at -20C or -80C until needed. This material is now ready for transport.

This procedure can be adapted for the following:
Toxocara – 2-5 per petri dish depending on size
Tapeworm *Dipylidium caninum* – 10-15 cm per petri dish

Safety: Dog parasites can cause zoonotic infections in humans . We recommend full PPE including gown, gloves and surgical mask. In addition plastic apron and sleeve protectors are advised.


Workflow Summary
2hrs in 5x substrate
Decant substrate
Supernatant
5x ES
Transfer hookworms
Centrifuge 1000g/10 min/4°C
Freeze -20°C
16hrs in 2x substrate
Decant substrate
Supernatant
Day 1 ES
Day 1
Centrifuge 1000g/10 min/4°C
Freeze -20°C
16hrs in 2x substrate
Decant substrate
Supernatant
Day 2 ES
Day 2
Centrifuge 1000g/10 min/4°C
Freeze -20°C
16hrs in 2x substrate
Decant substrate
Supernatant
Day 3 ES
Day 3
Centrifuge 1000g/10 min/4°C
Freeze -20°C

Substrate	x5		x2	
1% Glutamax	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies)	470 mL	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies)	485 mL
	5% antimycotic/ antibiotic	25 ml anti/ anti	2% antimycotic/ antibiotic	10 ml anti/anti
	1% Glutamax.	5 ml Glutamax	1% Glutamax.	5 ml Glutamax
PBS	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies)	475 mL	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies)	490 mL
	5% antimycotic/ antibiotic	25 ml anti/ anti	2% antimycotic/ antibiotic	10 ml anti/anti
RPMI	RPMI 1640 (gibco, Life Technologies)	470 mL	RPMI 1640 (gibco, Life Technologies)	485 mL
	5% antimycotic/ antibiotic	25 ml anti/ anti	2% antimycotic/ antibiotic	10 ml anti/anti
	1% Glutamax.	5 ml Glutamax	1% Glutamax.	5 ml Glutamax
2g/L Glucose	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies)	475 mL	Dulbecco's Phosphate Buffered Saline (DPBS) (+) Calcium Chloride (+) Magnesium Chloride (gibco, Life Technologies)	490 mL
	5% antimycotic/ antibiotic	25 ml anti/ anti	2% antimycotic/ antibiotic	10 ml anti/anti
	2g/L glucose	1 g D-glucose	2g/L glucose	1 g D-glucose

Appendix B

LCMS 5600 Putative Peptide sequencing data

Putative amino acid sequence†	ID Match in NCBIprot and Swissprot databases	Match against *Ancylostoma caninum* genome (ORF 50)
VPLGGPDGGNGGH	No significant match	No significant match
DGTNVGGSSGTI	No significant match	No significant match
PHGKVFGMFLYEYAR	No significant match	No significant match
SSGPATLM	No significant match	No significant match
SYSMTGSTT	No significant match	No significant match
LGGGVGCPF	No significant match	No significant match
AHTETESKKFTTA	No significant match	No significant match
NGGGVGGAAHPSP	No significant match	No significant match
QHGEGPPPFLK	No significant match	No significant match
HSHHDHYDMPYAGNM	No significant match	No significant match
PGLGTGHGPATT	No significant match	No significant match
NPGLFTGKG	No significant match	No significant match
PDAGGGLTPSGPM	No significant match	No significant match
PGLGPGSGSGNPAV	No significant match	No significant match
CGHAAPF	No significant match	No significant match
YGNGT	No significant match	No significant match
HGGGSP	No significant match	No significant match
VAGGPGSSGML	No significant match	No significant match
LAPSAPAPTTNSPGK	No significant match	No significant match
EMMEAEDDEVDE	No significant match	No significant match
NPGLFTGKG	No significant match	No significant match
HASPDGPNLPPMSASMTT	No significant match	No significant match
PTYLGMGLHTGAQST	No significant match	No significant match
VPLGGDGGNCGH	No significant match	No significant match
GGLLKPLL	No significant match	No significant match
ALNHCSVQ	No significant match	No significant match
MIIKPQP	No significant match	No significant match
DKPKLIL	No significant match	No significant match
DVFLGMFLYEYAR	No significant match	No significant match
GGPEGPPPFLK	100% match to immune-dominant hypodermal antigen	100% match to immune-dominant hypodermal antigen

†Where Alanine=A, Cysteine=C, Aspartic Acid=D, Glutamic Acid=E, Phenylalanine=F, Glycine=G, Histidine=H, Isoleucine=I, Lysine=K, Leucine=L, Methionine=M, Asparagine=N, Proline=P, Glutamine=Q, Arginine=R, Serine=S, Threonine=T, Valine=V, Tryptophan=W, Tyrosine=Y

Appendix C

Bio21 Institute – Polar Metabolite Standards

ns1:Co	ns1:CompoundName	MY RTs	Quant Ion
1	Lactic acid 2TMS	6.75	147
2	Alanine 2TMS	7.51	116
3	Glycine_2TMS	7.65	102
4	Oxalic acid_2TMS	8.15	190
5	Isoleucine_1TMS	8.76	158
6	beta-alanine_2TMS	8.96	174
7	Malonic acid_2TMS	9.16	233
8	Valine,DL_2TMS	9.39	144
9	Benzoic acid	10.11	179
10	Urea_2TMS	10.13	189
11	Serine_2TMS	10.19	204
12	Leucine_2TMS	10.46	158
13	Thymidine(BP)_TMS	10.49	103
14	Hypoxanthine_2TMS	10.63	265
15	Isoleucine_2TMS	10.79	158
16	Threonine,DL_2TMS	10.86	117
17	Maleic acid_2TMS	11.04	245
18	Glycine_3TMS	11.11	248
19	Norleucine_2TMS	11.28	158
20	Succinic acid_2TMS	11.28	247
21	Fumaric acid_2TMS	11.91	245
22	Serine_3TMS	12.07	204
23	Threonine,DL_3TMS	12.46	291
24	Methionine_1TMS	13.12	176
25	beta-Alanine_3TMS	13.28	174
26	Aspartic acid_2TMS	13.29	232
27	Homoserine_3TMS	13.64	128
28	Erythrose,D_3TMS	13.67	161
29	Glutamine_3TMS_prod 1	14.14	156
30	Malic acid_3TMS	14.33	233
31	Nicotinamide (Vit. B3)_1TMS	14.43	-
32	Erythritol_4TMS	14.61	189
33	Putrescine_3TMS	14.77	174
34	Salicylic Acid_2TMS	14.77	267
35	Methionine_2TMS	14.92	176
36	Asparagine_3TMS	14.93	231
37	Proline, trans-4-hydroxyl-L-_3TMS	14.98	-
38	Pyroglutamic acid,DL_2TMS	15.44	156
39	Phenylalanine_1TMS	15.53	120
40	2-oxo-Glutaric acid_2TMS	15.88	-
41	Creatinine 2TMS	15.92	115
42	Glutamic acid 3TMS	16.61	246
43	Phenylalanine_2TMS	16.78	192
44	Tartaric acid_4TMS	16.81	292
45	Dodecanoic acid,n_1TMS	17.16	257
46	1,6-anhydro-beta-D-glucose	17.93	-
47	Lysine_3TMS_prod 1	18.01	-
48	Ribitol,D_5TMS	18.14	129
49	Glycerol 2-Phosphate_4TMS	18.24	-